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(54) Title: VECTORS FOR THE DIAGNOSIS AND TREATMENT OF SOLID TUMORS INCLUDING MELANOMA

(57) Abstract

The present invention is directed to the isolation and use of super-infective, tumor-specific vectors that are strains of parasites including, but not limited to, bacteria, fungi and protists. In certain embodiments, the parasites include, but are not limited to, the bacterium *Salmonella* spp., such as *Salmonella typhimurium*, the bacterium *Mycobacterium avium* and the protozoan *Leishmania amazonensis*. In other embodiments, the present invention is concerned with the isolation of super-infective, tumor-specific, suicide gene-containing strains of parasites for use in treatment of solid tumors.

VECTORS FOR THE DIAGNOSIS AND
TREATMENT OF SOLID TUMORS INCLUDING MELANOMA

This application is a continuation-in-part
5 application of United States Patent Application Serial No.
08/486,422 filed June 7, 1995, which is incorporated by
reference herein in its entirety.

1.   FIELD OF THE INVENTION

10   The present invention is concerned with the
isolation and use of super-infective, tumor-specific,
attenuated strains of parasites including, but not limited
to, bacteria, fungi and protists. In certain embodiments the
parasites include the bacterium *Salmonella* spp., such as
15 *Salmonella typhimurium*, the bacterium *Mycobacterium avium*,
and the protozoan *Leishmania amazonensis*, for the diagnosis
and treatment of sarcomas, carcinomas, and other solid tumor
cancers. In other embodiments, the present invention is
concerned with the isolation and use of super-infective,
20 tumor-specific, suicide gene-containing strains of parasites.

2.   BACKGROUND OF THE INVENTION

          Citation or identification of any reference in
Section 2 of this application shall not be construed as an
25 admission that such reference is available as prior art to
the present invention.
          A major problem in the chemotherapy of solid tumor
cancers is the delivery of therapeutic agents, such as drugs,
in sufficient concentrations to eradicate tumor cells while
30 at the same time minimizing damage to normal cells. Thus,
studies in many laboratories are directed toward the design
of biological delivery systems, such as antibodies,
cytokines, and viruses for targeted delivery of drugs, pro-
drug converting enzymes, and/or genes into tumor cells.
35 Houghton and Colt, 1993, New Perspectives in Cancer Diagnosis
and Management 1: 65-70; de Palazzo,et al., 1992a, Cell.
Immunol. 142:338-347; de Palazzo et al., 1992b, Cancer Res.

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52: 5713-5719; Weiner, et al., 1993a, J. Immunotherapy
13:110-116; Weiner et al., 1993b, J. Immunol. 151:2877-2886;
Adams et al., 1993, Cancer Res. 53:4026-4034; Fanger et al.,
1990, FASEB J. 4:2846-2849; Fanger et al., 1991, Immunol.
5 Today 12:51-54; Segal, et al., 1991, Ann N.Y. Acad. Sci.
636:288-294; Segal et al., 1992, Immunobiology 185:390-402;
Wunderlich et al., 1992; Intl. J. Clin. Lab. Res. 22:17-20;
George et al., 1994, J. Immunol. 152:1802-1811; Huston et
al., 1993, Intl. Rev. Immunol. 10:195-217; Stafford et al.,
10 1993, Cancer Res. 53:4026-4034; Haber et al., 1992, Ann. N.Y.
Acad. Sci. 667:365-381; Haber, 1992, Ann. N.Y. Acad. Sci.
667: 365-381; Feloner and Rhodes, 1991, Nature 349:351-352;
Sarver and Rossi, 1993, AIDS Research & Human Retroviruses
9:483-487; Levine and Friedmann, 1993, Am. J. Dis. Child
15 147:1167-1176; Friedmann, 1993, Mol. Genetic Med. 3:1-32;
Gilboa and Smith, 1994, Trends in Genetics 10:139-144; Saito
et al., 1994, Cancer Res. 54:3516-3520; Li et al., 1994,
Blood 83:3403-3408; Vieweg et al., 1994, Cancer Res. 54:1760-
1765; Lin et al., 1994, Science 265:666-669; Lu et al., 1994,
20 Human Gene Therapy 5:203-208; Gansbacher et al., 1992, Blood
80:2817-2825; Gastl et al., 1992, Cancer Res. 52:6229-6236.

   Because of their biospecificity, such systems could
in theory deliver therapeutic agents to tumors. However, it
has become apparent that numerous barriers exist in the
25 delivery of therapeutic agents to solid tumors that may
compromise the effectiveness of antibodies, cytokines, and
viruses as delivery systems. Jain, 1994, Scientific American
7:58-65 (Jain). For example, in order for chemotherapeutic
agents to eradicate metastatic tumor cells, they must
30   a) travel to the tumors via the vasculature;
    b) extravasate from the small blood vessels supplying
      the tumor;
    c) traverse through the tumor matrix to reach those
      tumor cells distal to the blood supply; and
35   d) interact effectively with the target tumor cells
      (adherence, invasion, pro-drug activation, etc).

Although antibodies and viruses can express
specific recognition sites for tumor cells, they are
dependent solely upon the forces of diffusion and convection
in order to reach these sites. According to Jain:

5   An agent that destroys cancers cells in a
                   culture dish should, in theory, be able
                   to kill such cells in the body....
                   Sadly, however, the existing
                   pharmacopoeia has not markedly reduced
10   the number of deaths caused by the most
                   common solid tumors in adults, among them
                   cancers of the lung, breast, colon,
                   rectum, prostate, and brain.... Before a
                   blood-borne drug can begin to attack
15   malignant cells in a tumor, it must
                   accomplish three critical tasks. It has
                   to make its way into a microscopic blood
                   vessel lying near malignant cells in the
                   tumor, exit from the vessel into the
20   surrounding matrix (the interstitium),
                   and finally, migrate through the matrix
                   to the cells. Unfortunately, tumors
                   often develop in ways that hinder each of
                   these steps.

25 Jain points out that blood vessels supplying tumors are
   irregular and convoluted in shape so that blood flow is
   frequently restricted compared to that in normally
   vascularized tissue. In addition, there is an unusually high
   interstitial pressure in many tumors that counteracts the
30 blood flow. Jain further points out that the two chief
   forces governing the transport of agents to tumor cells via
   the circulatory system are convection (the transport of
   molecules by a stream of flowing fluid), and diffusion (the
   movement of molecules from an area of high concentration to
35 an area of low concentration). Since tumors are often non-
   uniformly vascularized, many cells in the tumors receive
   nutrients through the process of diffusion through the

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matrix. Jain and coworkers obtained data suggesting that "a
continuously supplied monoclonal antibody having a molecular
weight of 150,000 daltons could take several months to reach
a uniform concentration in a tumor that measured one
5 centimeter in radius and had no blood supply in its center."

2.1. BACTERIAL INFECTIONS AND CANCER

Regarding bacteria and cancer, an historical review
reveals a number of clinical observations in which cancers
10 were reported to regress in patients with bacterial
infections. Nauts et al., 1953, Acta Medica. Scandinavica
145:1-102, (Suppl. 276) state:

> The treatment of cancer by injections of
> bacterial products is based on the fact
> 15   that for over two hundred years neoplasms
> have been observed to regress following
> acute infections, principally
> streptococcal. If these cases were not
> too far advanced and the infections were
> 20   of sufficient severity or duration, the
> tumors completely disappeared and the
> patients remained free from recurrence.

Shear, 1950, J. A.M.A. 142:383-390 (Shear), observed that 75%
of the spontaneous remissions in untreated leukemia in the
25 Children's Hospital in Boston occurred following an acute
episode of bacterial infection. Shear stated:

> Are pathogenic and non-pathogenic
> organisms one of Nature's controls of
> microscopic foci of malignant disease,
> 30   and in making progress in the control of
> infectious diseases, are we removing one
> of Nature's controls of cancer?

Subsequent evidence from a number of research
laboratories indicated that at least some of the anti-cancer
35 effects are mediated through stimulation of the host immune
system, resulting in enhanced immuno-rejection of the cancer
cells. For example, release of the lipopolysaccharide (LPS)

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endotoxin by Gram negative bacteria such as *Salmonella*
triggers release of tumor necrosis factor, TNF, by cells of
the host immune system, such as macrophages, Christ et al.,
1995, Science 268:80-83.   Elevated TNF levels in turn
5 initiate a cascade of cytokine-mediated reactions which
culminate in the death of tumor cells.   In this regard,
Carswell et al., 1975, Proc. Natl. Acad. Sci. USA 72:3666-
3669, demonstrated that mice injected with bacillus Calmette-
Guerin (BCG) have increased serum levels of TNF and that TNF-
10 positive serum caused necrosis of the sarcoma Meth A and
other transplanted tumors in mice.   Further, Klimpel et al.,
1990, J. Immunol. 145:711-717, showed that fibroblasts
infected *in vitro* with *Shigella* or *Salmonella* had increased
susceptibility to TNF.
15   As a result of such observations as described
above, immunization of cancer patients with BCG injections is
currently utilized in some cancer therapy protocols.   See
Sosnowski, 1994, Compr. Ther. 20:695-701; Barth and Morton,
1995, Cancer 75 (Suppl. 2):726-734; Friberg, 1993, Med.
20 Oncol. Tumor. Pharmacother. 10:31-36 for reviews of BCG
therapy.

2.2. **PARASITES AND CANCER CELLS**
          Although the natural biospecificity and
25 evolutionary adaptability of parasites has been recognized
for some time and the use of their specialized systems as
models for new therapeutic procedures has been suggested,
there are few reports of, or proposals for, the actual use of
parasites as vectors.
30   In this regard, Pidherney et al., 1993, Cancer
Letters 72:91-98 (Pidherney et al.) and Alizadeh et al.,
1994, Infect. Immun. 62:1298-1303 (Alizadeh et al.) have
provided evidence that the pathogenic free-living amoeba,
Acanthamoeba castellani, has tumorcidal capabilities toward
35 human tumor cells, including melanoma, when added to tumor
cells growing in culture or when injected directly into
tumors in nude mice.   Pidherney et al. conclude:

The feasibility of utilizing the
tumorcidal properties of pathogenic/free-
living amoebae and their cell-free
products in the treatment of drug-
5   resistant or radio-resistant tumors
warrants further investigation.
However, Pidherney et al. also point out that such
pathogenic/free living amoebae can exist either as free-
living organisms feeding on bacteria or as opportunistic
10 pathogens producing life-threatening meningoencephalitis or
blinding keratitis.

Thus, it is readily apparent that for any parasite
to be effective as a therapeutic vector, for example, for
human tumors, the benefit of the parasite as a vector must
15 outweigh its risk as a pathogen to the patient. Therefore,
although Pidherney et al. and Alizadeh et al. demonstrated
cytotoxicity of pathogenic amoebae toward tumor cells, and
further suggested their use in the treatment of drug-
resistant and radio-resistant tumors, they offered no
20 solution for the inherent pathogenicity of these organisms
once injected into cancer patients. Furthermore, they
offered no method, e.g., genetic selection for isolating
super-infective, tumor-specific strains of pathogenic amoebae
nor did they suggest insertion into the amoebael genome of
25 genetic constructs containing inducible genes for the
synthesis and secretion of pro-drug converting enzymes and/or
suicide gene products.

Likewise, Lee et al., 1992, Proc. Natl. Acad. Sci.
USA 89:1847-1851 (Lee et al.) and Jones et al., 1992, Infect.
30 Immun. 60:2475-2480 (Jones et al.) isolated mutants of
Salmonella typhimurium that were able to invade HEp-2 (human
epidermoid carcinoma) cells in vitro in significantly greater
numbers than the wild type strain. The "hyperinvasive"
mutants were isolated under conditions of aerobic growth of
35 the bacteria that normally repress the ability of wild type
strains to invade HEp-2 animal cells. However, Lee et al.
and Jones et al. did not suggest the use of such mutants as

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therapeutic vectors, nor did they suggest the isolation of
tumor-specific bacteria by selecting for mutants that show
infection preference for melanoma or other cancers over
normal cells of the body. Without tumor-specificity or other

5 forms of attenuation, such hyperinvasive *Salmonella*
typhimurium as described by Lee et al. and Jones et al. would
likely be pan-invasive, causing wide-spread infection in the
cancer patient. Further, without selection for tumor
specificity or employment of other forms of attenuation, use

10 of such bacteria as therapeutic vectors would increase the
risk of pan-infection and septic shock to the cancer patient.

Pan et al., 1995, Nature Medicine 1:471-477 (Pan et
al.) described the use of *Listeria monocytogenes* as a vaccine
for the immunization of mice against lethal challenges with

15 tumor cells expressing the same antigen expressed by the
Listeria vaccine. In addition, they showed regression of
established tumors when immunized after tumor development in
an antigen specific T-cell-dependent manner. However, Pan et
al. did not show that *Listeria monocytogenes* could be used as

20 a tumor specific vector, which would target and amplify
within the tumor. Rather, Pan et al. showed that recombinant
Listeria monocytogenes has the ability to deliver a foreign
antigen to the immune system and to involve cell-mediated
immunity against the same antigen.

25 Sizemore et al., 1995, Science 270:299-302
(Sizemore et al.) described the use of attenuated *Shigella*
bacteria as a DNA delivery vehicle for DNA-mediated
immunization. Sizemore et al. showed that an attenuated
strain of *Shigella* invaded mammalian cells in culture and

30 delivered DNA plasmids containing foreign genes to the
cytoplasm of the cells. Foreign protein was produced in the
mammalian cells as a result of the procedure. The *Shigella*
vector was designed to deliver DNA to colonic mucosa,
providing a potential oral and mucosal DNA immunization

35 procedure as well as other gene immunotherapy strategies.
However, Sizemore et al. did not suggest the use of such
attenuated *Shigella* as tumor vectors in that they could be

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used to target tumors and thereby express genes within them.
Rather, Sizemore et al. envisioned its use in vaccination
therapy following oral delivery and invasion of the mucosa.

  Clostridium was previously investigated as a
5 potential therapeutic vector for solid tumors. The
propensity of spores of the obligate anaerobe *Clostridium* to
germinate in necrotic tissues is well known. Tetanus and gas
gangrene result from successful colonization of necrotic
tissue by pathogenic members of this genus.

10   Parker et al., 1947, Proc. Soc. Exp. Biol. Med. pp.
461-467 first showed that direct injection of spores of
Clostridium histolyticus into a transplantable sarcoma
growing in a mouse caused oncolysis, *i.e.*, liquification, as
well as regression of the tumor. In general the process of
15 *Clostridium*-mediated oncolysis was accompanied by acute
toxicity and death of the mice. Malmgren and Flanigan, 1955,
Cancer Res. 15:473 demonstrated that mice bearing mammary
carcinomas, hepatomas, and other tumors died within 48 hrs of
intravenous injection of *Clostridium tetani* spores, whereas
20 control, non-tumor bearing animals were asymptomatic for 40
days. Möse and Möse, 1964, Cancer Res. 24:212-216 (Möse and
Möse) described the colonization and oncolysis of tumors by
Clostridium butyricum, strain M-55, a non-pathogenic soil
isolate. Möse and Möse established the lack of human
25 pathogenicity of the M-55 strain by administering spores to
themselves, as reported by Carey et al., 1967, Eur. J. Cancer
3:37-46. Using *Clostridium butyricum* strain M-55, Möse and
Möse reported that intravenous injections of spores caused
oncolysis of the mouse Erlich ascites tumor, growing
30 experimentally as a solid tumor. Aerobic spore-forming
organisms--*e.g.*, *Bacillus mesentericus*, *Bacillus subtilis*,
which were prepared in a similar manner, did not show any
oncolysis under the same conditions. Möse and Möse concluded
that the clostridial oncolysis was restricted to anaerobic
35 areas of the tumors because of the anaerobic metabolic
requirements of the bacteria.

Gericke and Engelbart, 1964, Cancer Res. 24:217-221
showed that intravenously injected spores of strain M-55
produced extensive lysis of a number of different tumors, but
with shortened survival times of the *Clostridium*-treated,

5 tumor-bearing animals compared to non-treated tumor-bearing
animals. Further, they found that "metastases in organs or
lymph nodes were unaffected by the spores unless the
metastatic tumors had reached a considerable size."

Thiele et al., 1964, Cancer Res. 24:222-233 showed

10 that intravenously injected spores of a number of species of
nonpathogenic *Clostridia*, including M-55, localized and
germinated in tumor tissue, but not in normal tissues of the
mouse. Thiel et al., 1964, Cancer Res. 24:234-238 found that
spore treatment produced no effect when administered early in

15 the development of the tumor, *i.e.*, when the tumors were of
small size. While the spores caused oncolysis in tumors of
sufficient size, there was no effect in smaller tumors or
metastases. The animals regularly died during oncolysis.
Carey et al., 1967, Eur. J. Cancer 3:37-46, concluded that

20 small tumors and metastases had been noted to be resistant to
oncolysis whereas large neoplasms were particularly
favorable. Thus, the qualitative differences in germination
of spores were likely to be not a characteristic of
neoplastic and normal tissues per se, but related to

25 physiologic and biochemical conditions found within large
tumor masses.

Recent molecular genetic studies have focused on
anaerobic bacteria of the genus *Clostridium* as potential
tumor vectors. Fox et al., 1996, Gene Therapy 3:173-178

30 using a *Clostridium* expression vector were able to transform
the *E. coli* cytosine deaminase gene into *Clostridium*
beijerincki, which resulted in increased cytosine deaminase
activity in the growth medium supernatant and cell extracts
of transformed clostridial bacteria. Such supernatants, when

35 added to cultures of mouse EMT6 carcinoma made the cells
sensitive to 5-fluorocytosine, presumably through its
conversion to the toxic 5-fluorouracil. Similarly, Minton et

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al., 1996, FEMS Microbiol. Rev. 17:357-364 inserted the *E. coli* nitroreductase gene into *Clostridium beijerincki* and were able to detect expression of the gene in an *in vivo* murine tumor model through the use of antibodies directed

5   against the *E. coli* nitroreductase gene. The nitroreductase gene product activates CB1954, a potent alkylating agent.

Nothing in any of the above references (or any other references known to the present inventors) suggests the use of any microorganisms, other than the obligate anaerobe

10 *Clostridium*, as a potential therapeutic vector for solid tumors.

2.3. **ATTENUATED *SALMONELLA* SPP.**

Bacon et al., 1950, Br. J. Exp. Path. 31:703-713;

15 Br. J. Exp. Path. 31:714-724; 1951, Br. J. Exp. Path. 32:85-96 demonstrated that attenuation of *Salmonella* for virulence in mice can be achieved through auxotrophic mutations, *i.e.*, through the use of mutants which lack the ability to synthesize precursor molecules necessary for growth. More

20 specifically, the authors showed that purine-requiring (Pur⁻) auxotrophs of *Salmonella* were attenuated in mice.

Hoiseth and Stocker, 1981, Nature 291: 238-239 showed that *Salmonella typhimurium* auxotrophic mutants with requirements for aromatic amino acids (Aro⁻) were attenuated

25 for virulence in C57BL mice. Further, Su et al., 1992, Microbiol. Pathogenesis 13:465-476 showed that one such Aro⁻ mutant, the attenuated antigen carrier strain of *Salmonella typhimurium*, SL3261, was useful as a vaccine. The Shiga toxin B-subunit/hemolysin A (C-terminus) fusion protein was

30 expressed and underwent extracellular export resulting in antigen-specific immune responses in mice inoculated with these bacteria.

O'Callaghan et al., 1988, Infect. Immun. 56:419-423 characterized *Salmonella typhimurium* that were both Aro- and

35 Pur- and found that although they were highly attenuated in BALB/c mice, they persisted for several weeks in the livers and spleens following i.v. injections. They were found to be

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ineffective as vaccines when administered either orally or i.v.

Johnson et al., 1991, Mol. Microbiol. 5:401-407 (Johnson et al.) demonstrated that attenuation in *Salmonella*
5 virulence can be achieved through mutations in the heat shock inducible protein HtrA, a serine protease. Chabalgoity et al., 1996, Mol. Microbiol. 19:791-801, demonstrated that such attenuated htrA- *Salmonella typhimurium* were useful as live vaccines.

10   However, none of the references by Bacon et al., Hoiseth and Stocker, O'Callaghan et al., Johnson et al., Su et al. 1992, Chabalgoity et al. 1996, nor any of the studies referred to in Table 4, *infra*, suggest that such avirulent strains of *Salmonella typhimurium* would survive and
15 proliferate within solid tumors, nor that such avirulent mutants might be used as vectors for solid tumor therapy.

2.4. **OBJECTIVES OF THE INVENTION**

The problems associated with the many physical
20 barriers for delivery of therapeutic agents to solid tumors provide clear and difficult obstacles in the design of effective delivery systems. Thus, there has been a long felt need in the art to provide delivery systems which are able to overcome these obstacles.

25   It is an object of the present invention to use and to provide more advanced biological vectors such as parasites having several distinct advantages as a novel delivery system, some of which are listed below, as well as to meet the challenges of tumor therapy.

30   Antibiotic Sensitivity: It is an advantage for a tumor-specific parasitic vector to be sensitive to exogenously administered antibiotics. Parasites, such as bacteria, can be eradicated within their hosts by the administration of antibiotics. Such antibiotic sensitivity
35 allows for the eradication of the parasite from the cancer patient's body upon completion of the therapeutic protocol.

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Biospecificity: It is an advantage for a vector to express specificity for its target cell, *e.g.*, a tumor cell. The more specificity, of the vector for the tumor cell, the lower the inoculum necessary for effective therapy, thereby

5 reducing the risk of septic shock or pan-infection to the cancer patient. Parasites show a great degree of natural biospecificity, having evolved to utilize a variety of specific recognition and invasion mechanisms. (For general discussions on biospecificity see: Falkow, 1991, Cell

10 65:1099-1102; Tumomanen, 1993, Am. Soc. Microbiol. 59:292-296).

Mutant Isolation and Genetic Manipulation: It is an advantage, in the design and isolation of a parasite as a tumor-specific, therapeutic vector, for the parasite to be

15 amenable to genetic manipulation. Parasites with haploid genomes and short generation times, for example, bacteria such as *Salmonella typhimurium* and enteroinvasive *Escherichia coli*, can be readily subjected to mutagenesis followed by enrichment procedures for the isolation of strains with

20 desired new characteristics (see generally, Neidhardt et al., (ed.) 1987, *Escherichia coli* and *Salmonella typhimurium*, Cellular and Molecular Biology. American Society for Microbiology, pp 990-1033. Furthermore, the methods for the genetic analysis and stable introduction of genetic

25 constructs into these bacteria are well known to the science of molecular genetics.

Chemotaxis: A chemotactic response toward cancer cells is an advantage for a tumor-specific vector, for example as a stimulus for the vector to invade through a

30 basement membrane matrix such as that produced by endothelial cells in the vasculature, or as a stimulus for the vector to seek out cancer cells surrounded by tumor matrix. Chemotactic responses in parasites and commensalists or mutualists, particularly in bacteria such as *Escherichia coli*

35 and *Salmonella typhimurium*, are well documented. For a review of chemotaxis see Macnab, 1992, Ann. Rev. Genet. 26:131-158.

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Replication Within Target Cells: The ability to replicate within target cells is an advantage for a tumor-specific vector. Such an ability allows for amplification of the therapeutic vector number within the infected cancer
5 cell, thus increasing the therapeutic effectiveness of the vector. Progeny of vectors within cancer cells further infect surrounding or distant cancer cells, thus amplifying the vector number within the tumor cell population.

Anaerobic and Aerobic Metabolism: The ability to
10 express invasive and amplification capacities under either aerobic or anaerobic conditions is an advantage for a tumor-specific vector. Solid tumors generally contain vascularized, oxygen-rich areas as well as necrotic oxygen-poor areas. A vector that is functional in both such
15 environments would be able to reach a larger portion of tumor cells than one that can function in only one environment, such as, for example, an obligate anaerobe or aerobe.

3.   **SUMMARY OF THE INVENTION**

20   The present invention provides compositions and methods for delivery of genes and/or gene products to and/or into target mammalian cells *in vitro* or *in vivo*. The genes and/or gene products are delivered by microorganism vectors, including bacteria, fungal and protozoan parasites, which are
25 selected and/or genetically engineered to be specific to a particular type of target mammalian cell. In a preferred embodiment, the vectors function under both aerobic and anaerobic conditions, are super-infective, tumor-specific microorganisms useful for diagnosis or treatment of sarcomas,
30 carcinomas, lymphomas or other solid tumor cancers, such as germ line tumors and tumors of the central nervous system, including, but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma,
35 glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma.

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Vectors useful for the methods of the present invention include but are not limited to *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella pneumophila*,
5 *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Streptococcus* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, BCG, *Mycoplasma hominis*,
10 *Rickettsiae quintana*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp.,
15 *Trypanasoma cruzi*, *Encephahtozoon cuniculi*, *Nosema helminthorum*, *Unikaryon legeri*.

As used herein, *Salmonella typhimurium* encompasses all *Salmonella* species. It has long been recognized that the various "species" of the genus *Salmonella* are in fact a
20 single species by all acceptable criteria of bacterial taxonomy. The single species is now designated "*Salmonella enterica*". F. Neidhardt (ed.), *Escherichia coli* and *Salmonella*, 1996, Volume I, pp. *xx*, ASM Press, Washington DC.

An embodiment of the present invention is to
25 provide methods for the isolation of super-infective, attenuated, tumor-specific mutants of microorganisms such as bacterial, fungal and protozoan parasites. Further, the present invention provides methods for use of these microorganisms in the diagnosis and treatment of malignant
30 and/or metastatic solid tumor cancers, such as melanoma or colon cancer. Moreover, these mutant parasites may express specific gene products, some of which are secreted into the cytoplasm or vacuolar space of the infected cell.

The present invention provides methods for the
35 isolation of super-infective target cell-specific microorganisms. In particular embodiments, the invention provides for the isolation and use of super-infective, tumor-

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specific strains of parasites such as the bacterium
Salmonella spp., including *S. typhimurium*, the bacterium
Mycobacterium avium, and the protozoan *Leishmania*
amazonensis. The tumor-specific vectors can also contain
5 suicide genes.

One embodiment of the present invention provides
methods for the isolation of and compositions comprising
super-infective, tumor-specific mutants of *Salmonella* spp.,
e.g., *Salmonella typhimurium*, and for their use in the
10 diagnosis and treatment of sarcomas, carcinomas, melanomas,
colon cancer, and other solid tumor cancers. Another
embodiment of the present invention provides methods for the
isolation of and compositions comprising super-infective,
tumor-specific mutants of *Salmonella* spp. containing a
15 suicide gene. In a specific embodiment, the suicide gene is
thymidine kinase from *Herpes simplex* virus or cytosine
deaminase from *Escherichia coli* or human microsomal p450
oxidoreductase.

Another embodiment of the present invention
20 provides methods for the isolation of and compositions
comprising super-infective, tumor-specific mutants of the
protozoan, *Leishmania amazonensis* and for their use in the
diagnosis and treatment of sarcomas, carcinomas, melanomas,
colon cancer, and other solid tumor cancers.

25 Yet another embodiment of the present invention
provides methods for the isolation of and compositions
comprising super-infective, tumor-specific mutants of the
bacterium *Mycobacterium avium* and for their use in the
diagnosis and treatment of sarcomas, carcinomas, melanomas,
30 colon cancer, and other solid tumor cancers.

Yet another embodiment of the present invention
provides methods for attenuation of parasite vector toxicity
so as to reduce the risk of septic shock or other
complications in the host, *i.e.*, the patient receiving
35 vector-delivered gene therapy. Such methods include
mutagenesis of parasites; isolation of parasite mutants with
increased tumor specificity, increased specificity for

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suicide gene expression and concomitant reduced ability to
infect normal host cells in the body; isolation of mutants
with enhanced chemotactic abilities toward cancer cell
secretory products; isolation of mutants with genetically
5 altered lipopolysaccharide composition; and isolation of
mutants with altered virulence genes so as to achieve
specific survival of the parasitic vector in cancer cells as
opposed to normal cells of the host body.

          The present invention further encompasses use of
10 microorganism vectors for diagnosis or treatment of solid
tumor cancers.

          The present invention may be understood more fully
by reference to the following definitions, detailed
description of the invention, illustrative examples of
15 specific embodiments and the appended figures in which:

          4.   **DEFINITIONS**

          Attenuation: Attenuation, in addition to its
                                      traditional definition in which a
20   microorganism or vector is modified
                                      so that the microorganism or vector
                                      is less pathogenic, is intended to
                                      include also the modification of a
                                      microorganism or vector so that a
25   lower titer of that microorganism or
                                      vector can be administered to a
                                      patient and still achieve comparable
                                      results as if one had administered a
                                      higher titer of the parental
30   microorganism or vector. The end
                                      result of attenuation is that the
                                      risk of toxicity as well as other
                                      side-effects is decreased, when the
                                      microorganism or vector is
35   administered to the patient.

          Suicide gene: A suicide gene is defined as a gene
                                     that when delivered to a target cell

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and expressed by a vector of the present invention causes the death of the target cell and/or the vector.

5   Super-infective: A super-infective vector is defined as a vector which is able to attach and/or infect a target cell more readily as compared to the wild type vector. Depending on the population

10   density of the inoculum, the ratio between super-infective vectors and wild type vectors detectably infecting a target cell approaches 4:1, preferably 30:1, more

15   preferably 90:1. Most preferably, one is able to reduce the inoculum size and infection time so that only the super-infective vectors have time to attach to and/or infect

20   cancer cells growing in cell culture *in vitro* or as tumors *in vivo*.

Tumor-specific: A tumor-specific vector is defined as a vector which is able to distinguish between a cancerous

25   target cell and the non-cancerous counterpart cell so that the vector preferentially attaches to, infects and/or remains viable in the cancerous target cell.

30

5.   **BRIEF DESCRIPTION OF THE DRAWINGS**

   Figure 1. Figure 1 depicts a DNA cassette system for expressing pro-drug converting enzymes. Each of the components is generated by PCR using primers containing

35 specific restriction endonuclease sites *NotI*, *NsiI*, *NcoI*, *SfiI* or *PacI* that allow for simple interchange of individual components. For example, (A) is the coding sequence for pro-

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drug converting enzymes such as thymidine kinase, cytosine
deaminase or human microsomal p450 oxidoreductase; (B) is a
promoter, which is active in an inducible, constitutive or
cell specific manner; (C) is a N-terminal secretion signal
5   sequence, such as the β-lactamase signal sequence; and (D) is
a C-terminal secretion signal sequence, such as the
enteroinvasive *E. coli* hemolysin A signal sequence.

Figure 2A-B. Figure 2A-B are photomicrographs of
Salmonella typhimurium wild type strain ATCC No. 14028
10 infecting human melanoma cell line M2. A starting population
of ATCC No. 14028 was subjected to 10 cycles of infection
into and recovery from M2 melanoma cells before use in the
infection assay shown in Figure 2A-B. Figure 2A. Light
micrograph of an infected melanoma cell. Figure 2B. DAPI
15 staining of the cell showing cell nucleus, (n), and numerous
bacteria inside the cell, (arrow).

Figure 3A-C. Figure 3A-C are photomicrographs of
Salmonella typhimurium wild type strain ATCC No. 14028 during
the process of internalization into human melanoma cell line
20 M2. Figure 3A. Phase contrast micrograph of a host cell.
Figure 3B. DAPI staining of the host cell showing the
position of the bacteria, (arrow), and the host cell nucleus,
(n). Figure 3C. Lysosomal glycoprotein LAMP-1 antibody
staining of the host cell showing co-localization of the
25 bacteria with lysosomes and/or melanosomes.

Figure 4A-4F relate to producing converting enzyme
expression constructs and expression using the same.

Figure 4A-B. Expression of the *Herpes simplex*
thymidine kinase gene containing a β-lactamase secretory
30 signal sequence in *Salmonella typhimurium* super-infective
clone 72. Figure 4A. Immunoblot analysis of *Salmonella
typhimurium* strains using an anti-TK monoclonal antibody.
Lane 1: bacteria containing only the plasmid vector p279;
Lane 2: strain 14028 wt (CDC6516-60) (MO) containing the
35 cytoplasmicly expressed form of TK (pHETK2); Lane 3: strain
14028 clone 72 containing the cytoplasmicly expressed form of
TK (pHETK2); Lane 4: strain 14028 wt (MO) containing the β-

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lactamase fusion form of TK (p5-3); Lane 5: strain 14028
clone 72 containing the β-lactamase fusion form of TK (p5-3);
Lane 6: strain 14028 wt (MO) containing the β-lactamase
fusion form of TK (p21A-2); Lane 7: strain 14028 clone 72
5   containing the β-lactamase fusion form of TK (p21A-2).
Relative molecular mass x 10^3 is shown on the left. No
antibody reactivity is seen in the "vector only" control
(Lane 1). In each lane where the cytoplasmically expressed
TK is present (Lanes 2 and 3) two major isoforms of the
10   protein are seen; a higher molecular mass isoform containing
a leader sequence and a lower molecular mass isoform wherein
the leader sequence has been proteolytically cleaved off. In
each lane where the bacteria express the TK gene β-lactamase
signal sequence fusion (Lanes 4 to 7) two major isoforms of
15   the protein are also seen: a higher molecular mass form
containing the signal sequence and a lower molecular mass
isoform wherein the signal sequence has been proteolytically
cleaved off, which is the same apparent molecular mass as the
processed form of the cytoplasmic enzyme.
20   Figure 4B. Relative TK enzyme activity associated
with each of the samples in Figure A. Enzyme activity is
expressed as the total number of counts of ^{125}IdC
phosphorylated in a standard assay, Summers and Summers,
1977, J. Virol. 24:314-318. A small background is present in
25   a bacterial extract from the vector only sample (Lane 1).
Significantly higher levels of TK activity are observed in
the wild type and the super-infective clone 72 containing the
cytoplasmic form of TK pHETK2, Lanes 2 and 3. Similar levels
are observed in both the wild type and super-infective clone
30   72 containing the β-lactamase signal sequence fusion isoform
of TK p5-3, Lanes 4 and 5. Lower levels are observed in both
the wild type and super-infective clone 72 containing the β-
lactamase signal sequence fusion isoform of TK p21A-2, Lanes
6 and 7.
35   Figure 4-C is a schematic of the different *Herpes
Simplex Virus* thymidine kinase secretion and expression
constructs.

Figure 4-D is a schematic of the different human microsomal cytochrome p450 oxidoreductase expression constructs.

Figure 4-E is a schematic of the *E. coli* cytosine
5 deaminase secretion and expression construct.

Figure 4-F is a graph showing the amount of 5-FC converted to 5-FU by different bacteria.

Figure 5A-B. Figure 5A-B are photomicrographs of histologic sections from a Cloudman S91 melanoma/macrophage
10 hybrid #48 growing subcutaneously in a DBA/2J mouse. The tumor was excised from a mouse that had been inoculated 2 days earlier with 3 x 10^5 c.f.u. *Salmonella typhimurium* super-infective clone #72 carrying the *HSV* TK gene, clone #72^{5-3-2}. Figure 5A. A section stained with hematoxylin and eosin
15 shows tumor cells with a central area of necrosis, denoted by arrows. Figure 5B. A section stained with Brown-Brenn stain (tissue gram stain) shows gram negative bacteria in a necrotic area of the tumor, denoted by the arrow. When viewed under a light microscope, the bacteria stain
20 pink/purple against a yellow background.

Figure 6. Figure 6 is an electron micrograph of a section of a Cloudman S91 melanoma/macrophage hybrid #48 tumor excised from a DBA/2J mouse that had been inoculated i.p. 42 hours earlier with 4 x 10^6 *Salmonella typhimurium*
25 super-infective clone 72. Visible in the micrograph are two *Salmonella typhimurium* bacteria, denoted by arrows, along with numerous melanosomes (m), sub-cellular organelles characteristic of melanoma cells. The co-localization of *Salmonella* and such melanosomes indicates that the bacteria
30 are present in the cytoplasm of the melanoma cell. Magnification=21,000x.

Figure 7. Figure 7 is a photomicrograph of a histologic section from a B16F10 melanoma growing subcutaneously in a C57BL/6J mouse. The tumor was excised
35 from a mouse that had been inoculated 42 hours earlier with 1.8 x 10^5 c.f.u. *Salmonella typhimurium* super-infective clone #72 carrying the *HSV* TK gene. The sections were from the

same tumor examined with the electron microscope as detailed
in Figure 8. The section was stained with Brown-Brenn stain
(tissue gram stain) and shows gram negative bacteria in a
necrotic area of the tumor, denoted by arrows. When viewed
5 under a light microscope, the bacteria stain pink/purple
against a yellow background.

Figure 8. Figure 8 is an electron micrograph of a
section from a B16F10 melanoma tumor excised from a C57BL/6J
mouse that had been inoculated i.p. 42 hours earlier with 1.8
10 x 10^5 *Salmonella typhimurium* super-infective clone #72
carrying the *HSV* TK gene. The section was from the same
tumor examined with the light microscope as detailed in
Figure 7. The micrograph shows numerous *Salmonella
typhimurium* in extracellular spaces, denoted by arrows, and
15 in an area of necrosis. A single bacterium is also seen
within the cytoplasm of a dying melanoma cell. The cytoplasm
of the dying melanoma cell also contains numerous black
melanosomes (m), characteristic of the B16F10 melanoma.
Magnification = 9,750x.

20 Figure 9A-D. Figures 9A-D depict growth of
Cloudman S91 melanoma/macrophage hybrid #48 tumors in DBA/2J
mice under various treatment conditions. Mice were
inoculated s.c. in the flank region with 3 x 10^5 melanoma
cells. The tumors were palpable 8-10 days later, and some of
25 the mice were then further inoculated with *Salmonella
typhimurium* super-infective clone #72 carrying the *HSV* TK
gene. Twenty-four hours post inoculation with bacteria, some
groups of the mice were further inoculated i.p. with 2.0 mg
ganciclovir. Ganciclovir inoculations were repeated 6 times
30 over a 5 day period. Points represent caliper measurements
of tumors in 2 to 5 mice per treatment group at the days
indicated. Measurements in mm were made of length, width,
and height for each tumor and volumes were calculated in mm^3.
Average tumor volumes for each group of mice were defined as
35 100% on day 0, the beginning day of treatment. (Figure 9A).
Control mice: no *Salmonella*; no ganciclovir (Figure 9B).

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Ganciclovir only;   (Figure 9C). *Salmonella* only;   (Figure
9D). *Salmonella* + ganciclovir.

  Figure 10A-B. Figure 10A shows a control mouse and
Figure 10B shows a *Salmonella typhimurium*-infected (7B)

5 DBA/2J mouse. The mice were inoculated (s.c.) with Cloudman
S91 melanoma/macrophage hybrid #48 tumor cells. Upon the
appearance of palpable tumors some of the mice were
inoculated (i.p.) with 3 x 10^5 c.f.u. *Salmonella typhimurium*
clone 72 containing the *HSV* TK gene (clone #72^{5-3-2}), allowed

10 to eat and drink ad libitum for 10 days, and then treated
with Sulfatrim™ antibiotic in their drinking water for
several more days and photographed. Tumors in the depicted
mice were representative of the general state of tumor
progression in mice in *Salmonella*-treated and untreated

15 cages.

  Figure 11A-11H relate to the effects of
gancicylovir on tumor cell growth, *in vivo* or *in vitro*.

  Figure 11A-B. Figures 11A-B depict a control
(Figure 11A) and a *Salmonella typhimurium*-infected (Figure

20 11B) DBA/2J mouse. The mice were inoculated (s.c.) with
Cloudman S91 melanoma/macrophage hybrid #48 tumor cells.
Upon the appearance of palpable tumors, some of the mice were
inoculated with 3 x 10^5 c.f.u. *Salmonella typhimurium* clone
#72 containing the *HSV* TK gene (clone #72^{5-3-2}). Control and

25 Salmonella-infected mice were then injected (i.p.) with 2.0
mg ganciclovir a total of 5 times over a 4 day period. The
mice were then treated with Sulfatrim™ antibiotic in their
drinking water for several more days and photographed. The
depicted mice are representative of the general state of

30 tumor progression in mice, either in *Salmonella*-treated and
untreated cages.

  Figures 11(C-E) show the effect of ganciclovir on
the growth of B16F10 melanomas in mice with and without
inoculation of *Salmonella typhimurium* clone YS7211 (Figure

35 11-1 A); clone YS7213 (Figure 11-1 B); and clone YS7212
(Figure 11-1 C).

Figure 11-F is a graph showing the growth of B16F10 melanoma cells in monolayer culture in the presence or absence of ganciclovir at 10 μg/ml or 25 μg/ml.

Figure 11-G is a graph showing the effect of
5 ganciclovir on the growth of B16F10 melanomas in mice following inoculation of *Salmonella typhimurium* clone YS7211 carrying the *HSV* thymidine kinase gene, YS7211/p5-3, with and without ganciclovir.

Figure 11-H is a graph showing the effect of total
10 amounts of ganciclovir on the growth of B16F10 melanomas in mice following inoculation with *Salmonella typhimurium* clone YS7211 carrying the *HSV* thymidine kinase gene, YS7211/p5-3.

Figure 12A-B are electron micrographs. Figure 12A is an electron micrograph of a section from a HCT 116 human
15 colon tumor excised from a BALB/c *nu/nu* mouse. The mouse had been inoculated i.p. 72 hours earlier with 2.8 x 10^5 c.f u. *Salmonella typhimurium* super-infective clone #72 containing the *HSV* TK gene, clone #72^{5-3-2}. Shown in the micrograph are numerous *Salmonella typhimurium* within the cytoplasm of a
20 neutrophil associated with the tumor. Some of the bacteria are undergoing division as denoted by arrows. The neutrophil or polymorphonucleoleukocyte is characterized by its multi-lobed nucleus (n), (Magnification=21,000x).

Figure 12-B is an electron micrograph showing
25 numerous *Salmonella typhimurium*, denoted by arrows, in extracellular spaces as well as contained within a single cell, possibly a neutrophil, seen in the upper left. Also seen in the field are two unidentified cells that appear to be dying as indicated by the large intracellular space, along
30 with cellular debris.

Figure 13. Figures 13A-B depict *Leishmania amazonensis* adhesion to human melanoma cell line M2. Figure 13A. Phase contrast micrograph showing parasites attached to cell, (arrow). Figure 13B. DAPI staining showing the
35 parasite DNA, (arrows), and the host cell nucleus, (n).

Figure 14. Figures 14A-C are photomicrographs of *Leishmania amazonensis* during the process of internalization

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into human melanoma cell line M2. Figure 14A. Phase contrast of a *Leishmania* trypomastigote, (arrow), entering a host cell. Figure 14B. DAPI staining showing the position of the parasite, (arrow), and the host cell nucleus, (n).

5 Figure 14C. Lysosomal glycoprotein LAMP-1 antibody staining of the host cell showing co-localization of the bacteria and the lysosomes.

Figure 15 A-D. Figure 15 A-C are graphs showing growth of *Salmonella typhimurium* clone 72 and clone YS7212 in

10 minimal Medium 56 supplemented with glucose only; Medium 56 with glucose plus adenine, vitamin B1, isoleucine, valine, and uracil; or Medium 56 with tumor extract (10%) only.

Figure 15-D is a graph depicting growth of *Salmonella typhimurium* clones 72 and YS7212 following

15 invasion into human M2 melanoma cells in culture.

Figure 16 A-D are graphs showing growth of B16F10 melanomas in C57B6 mice with and without inoculation of *Salmonella typhimurium* strains YS721 (Figure 16-A); YS7213 (Figure 16-B); YS7211 (Figure 16-C); and YS7212 (Figure 16-

20 D).

Figure 17 is a graph showing that combination of CD and 5-fluorocytosine prolong the survival of animals bearing B16F10 lung metastases when the animals are infected with a tumor-specific vector carrying the cytosine deaminase

25 expression construct YS7212/pCD-Sec1.

Figure 18 is a graph showing TNF-α production by human macrophages incubated with lipopolysaccharide isolated from wild type and attenuated strains of *Salmonella typhimurium*.

30 Figure 19 is a bar graph demonstrating the effect of clones YS7211 and YS7212 expressing the *HSV* thymidine kinase gene, YS7211/p5-3 and YS7212/p5-3, respectively, on mice bearing metastatic B16F10 tumors with or without ganciclovir treatment.

35

6.   **DETAILED DESCRIPTION OF THE INVENTION**

The present invention is directed to the isolation
of novel therapeutic and diagnostic parasitic vectors for
solid tumor cancers, such as sarcomas, carcinomas, lymphomas
5   or other solid tumor cancers, for example, germ line tumors
and tumors of the central nervous system, including, but not
limited to, breast cancer, prostate cancer, cervical cancer,
uterine cancer, lung cancer, ovarian cancer, testicular
cancer, thyroid cancer, astrocytoma, glioma, pancreatic
10 cancer, stomach cancer, liver cancer, colon cancer, melanoma
and their use. Described, in detail below, are the novel
intracellular parasite vectors; methods for the isolation of
the novel vectors; genetic engineering of the isolated
vectors; and methods for use of the novel vectors as well as
15 other vectors in treatment or detection of solid malignant
tumors, including metastatic tumors and tumor cells.

6.1. **NOVEL VECTORS AND METHODS FOR THEIR ISOLATION**

The isolated vectors, which are for example,
20 bacteria, fungi or protista, are able to differentiate
between cancerous cells and non-cancerous counterpart cells.
For example, the isolated vectors are able to differentiate
melanoma cells from melanocytes or differentiate colon cancer
cells from normal colon epithelial cells. Table I is a
25 representative list, which is in no way meant to limit the
present invention, of intracellular parasitic and pathogenic
microorganisms which are useful as tumor-specific vectors for
the present invention and/or for isolation of novel mutant
strains which are super-infective and tumor-specific vectors
30 for use in the present invention.

35

- 25 -

TABLE 1

REPRESENTATIVE LIST OF ORGANISMS USEFUL AS VECTORS

Gram negative bacteria
5   *Borrelia burgdorferi*
           Brucella melitensis
           Escherichia coli
           enteroinvasive *Escherichia coli*
           Legionella pneumophila
           Salmonella typhi
           Salmonella typhimurium
10   *Shigella spp.*
           Treponema pallidum
           Yersinia enterocohtica

Gram positive bacteria
           BCG (*Bacillus Calmette-Guerin*)
           Chlamydia trachomatis
           Listeria monocytogenies
15   *Mycobacterium avium*
           Mycobacterium bovis
           Mycobacterium tuberculosis
           Mycoplasma hominis
           Rickettsiae quintana
           Streptococcus spp.

20 Fungi
           Cryptococcus neoformans
           Histoplasma capsulatum
           Pneumocystis carnii

Apicomplexans
25   *Eimeria acervulina*
           Neospora caninum
           Plasmodium falciparum
           Sarcocystis suihominis
           Toxoplasma gondii

Kinetoplastida
30   *Leishmania amazonensis*
           Leishmania major
           Leishmania mexacana
           Leptomonas karyophilus
           Phytomonas spp.
           Trypanasoma cruzi

Microsporidians
35   *Encephahtozoon cuniculi*
           Nosema helminthorum
           Unikaryon legeri

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The bacterium *Salmonella typhimurium*, the bacterium *Mycobacterium avium*, and the protozoan *Leishmania amazonensis* are each particularly useful vectors for the present invention, since each of these organisms shows natural

5   preference for attachment to and penetration into certain solid tumor cancer cells in tissue culture, as opposed to non-cancerous counterpart cells. Since these vectors, such as *Salmonella*, have a natural ability to distinguish between cancerous cells and their non-cancerous counterpart cells

10   they are directly applicable to the methods for diagnosis or treatment according to the present invention. However, this tumor-specific ability, as well as, the ability to be super-infective as compared to the "wild type" parent strain may be enhanced and selected for by using the methods of the present

15   invention described in Sections 6.1.1-6.1.4., *infra*.

6.1.1.   ISOLATION BY CYCLING THROUGH *IN VITRO* TISSUE CULTURE

One embodiment of the present invention is to

20   isolate the novel vectors of the present invention by cycling a microorganism through a pre-selected target cell, preferably a solid tumor cancer cell, with one or more cycles of infection in *in vitro* tissue culture so that the cycled population and/or clonal isolates therefrom demonstrate

25   enhanced infectivity of the target tumor cell as compared to the starting microbial population and enhanced selectivity as compared to the non-cancerous counterpart cell. The method entails selecting a parasite or pathogen and adding the microorganism to an *in vitro* tissue culture system of the

30   particular type of solid tumor that one wishes to use as a target cell. For example, if one desires to target melanoma tumors, the target cell may be M2 human melanoma cells. After incubating the tumor cells and microorganisms together, which allows enough time for the microorganism to attach

35   and/or infect the tumor cell, the tumor cell culture is washed with either buffer or medium which contains an antibiotic agent effective against the specific microorganism

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used.   The antibiotic agent kills any microorganisms that
have not attached to and infected the tumor cell.   If
desired, the infected tumor cell culture may be incubated
further in medium containing antibiotic for varying times,
5 depending on the type of population of microorganisms to be
isolated.   For example, for longer incubation times, the
microorganism population isolated has enhanced survival
and/or proliferative abilities inside the tumor cells as
compared to the starting population of microorganisms.
10 Additionally, the isolated populations can be cultured to
isolate single colony clones using standard techniques.

      The infected animal cells are collected and lysed,
thus freeing the internalized microorganisms.   The
microorganism can then be isolated, for example, by
15 centrifugation (2000x g for 4 minutes) and resuspending in
fresh medium.   The isolated microbial population may then be
used for additional cycles of infection into and isolation
out of the target tumor cell.   The isolated microbial
population may be placed first in appropriate growth medium
20 for 1-2 doubling times before being subjected to additional
infection cycles to insure their viability.   The isolated
microbial population may also be cultured so as to isolate
and collect single colony clones.   The isolated
microorganisms may also undergo known *in vitro* techniques to
25 determine their relative infective and selective abilities as
compared to the "wild type" parent strain which did not
undergo *in vitro* selection.   For example, in side by side
comparisons one may test the relative infectiveness of the
isolated microorganism as compared to the "wild type" by
30 using assays designed to quantitate the number of
microorganisms which have attached to or invaded the target
tumor cell and/or their ability to distinguish between
cancerous and non-cancerous cells.   In addition, parameters
such as microorganism population density, may be varied in
35 these *in vitro* assays which assists in determining what
effect the overall concentration of inoculum of the clone or
population being tested has on the ability of the

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microorganisms to differentiate between the target tumor
cells and their non-cancerous counterparts.

For an illustrative example of super-infective,
tumor-specific vectors isolated by cycling through *in vitro*
5 tissue culture, see Section 7, *infra*.

6.1.2.   **ISOLATION BY CYCLING THROUGH *IN VIVO***
SOLID TUMORS

Another embodiment of the present invention is to
10 isolate the novel vectors of the present invention by cycling
the microorganism through solid tumors *in vivo*. This
procedure is performed using experimental tumor models in
mammals such as, for example and not by way of limitation,
B16 mouse melanoma cells which form melanoma tumors in C57B6
15 mice and HCT116 human colon carcinoma cell which form colon
carcinomas in *nu/nu* and other immuno-compromised mice.
Additionally, fresh biopsies of tumor tissue which are
obtained surgically from a cancer patient may be used to
inoculate *nu/nu*, *scid* or other immuno-compromised mice.
20 These tumors in mice which have grown from inoculated cancer
cells are used as *in vivo* targets for the isolation of super-
infective and tumor-specific vectors in a similar manner as
in vitro target cells. Any tumor growing in mice or any
other animal may be used in the present invention as a target
25 for the isolation of super-infective and tumor-specific
vectors *in vivo*.

Once the tumor is established in the mouse, by, for
example, inoculation of cancer cells sub-cutaneously or
transplantation of a tumor mass, the selected microorganism
30 is inoculated into the mouse. After a pre-determined
infection time after inoculation in which the microorganism
becomes co-localized with the tumor and/or infects the tumor
cells, the mice are sacrificed, the tumors excised, weighed
and homogenized. An aliquot may be diluted into the proper
35 microorganism growth medium and incubated at the proper
growth conditions for 1-2 population doublings to insure the
recovery of viable microorganisms for successive inoculations

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into tumor bearing mice. Further, if the isolated population
is to undergo successive inoculations in tumor bearing mice,
upon each successive inoculation, the number of
microorganisms in the inoculate and the time of infection may
5 be reduced to increase the stringency of selection for tumor-
specific isolates. Additionally, the isolated populations
can be cultured to isolate single colony clones using
standard techniques. The isolated microorganisms may be used
also in *in vitro* assays to determine their relative infective
10 and selective abilities as compared to the "wild type" parent
strain which did not undergo an *in vivo* selection procedure.

For an illustrative example of super-infective,
tumor-specific vectors isolated *in vivo* in tumor-bearing
mice, see Section 9, *infra*.

15

6.1.3.   ISOLATION BY *IN VITRO* CHEMOTAXIS USING MEDIUM CONDITIONED BY THE TARGET TUMOR CELL

Another embodiment of the present invention is to
provide methods for isolating super-infective and/or tumor
20 cell-specific vectors by chemotaxis so that the isolated
microorganisms have increased chemotactic ability towards
tumor cell secretory products. The method entails using
capillary tubes which are loaded with either liquid control
medium or medium that has been conditioned by the target
25 tumor cell as described by Adler (1973, J. General
Microbiology 74:77-91). Conditioned medium is medium in
which the target cells have been grown and subsequently has
been filtered to remove the cells. One end of the capillary
is sealed in a flame; the capillary is then quickly passed
30 several times through a flame and is immediately plunged open
end down into a beaker containing either the conditioned or
control medium. As the capillaries cool, the liquid is drawn
up inside.

The loaded capillary tubes are inserted open end
35 down into a centrifuge tube containing medium and a
suspension of the pre-selected microorganism. After a pre-

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determined period of incubation at 37°C in which the
microorganism chemotacts into the capillary tubes, the
capillary tubes are removed with forceps, the sealed ends are
opened and the opened capillaries are transferred into
5 centrifuge tubes containing nutrient medium appropriate for
the particular microorganism. It is important that the upper
tips of the capillary tubes are covered with an appropriate
medium for the particular type of microorganism to assure
quantitative recovery of the microorganism from the capillary
10 tubes during centrifugation. The capillary tubes are
centrifuged, for example, at 4000x g for 4 minutes, to force
the microorganism out of the tube. The capillary tubes are
removed, the microorganism resuspended, and an aliquot spread
onto the appropriate medium in either solid or liquid form to
15 allow for quantitation.

Significant increases in the number of
microorganisms entering into the capillary tubes containing
conditioned medium as compared to controls indicates a
positive chemotactic response toward secreted products of the
20 target cell found in the conditioned medium. The populations
isolated by this in vitro technique can undergo successive
chemotaxis assay isolation or be used to isolate single
colony clones. These clones or populations can be compared
to the "wild type" parent strain in their ability to
25 distinguish between the target cancerous cell and the non-
cancerous counterpart cell as well as for super-infective
ability.

For an illustrative example of super-infective,
tumor-specific vectors isolated by *in vitro* chemotaxis using
30 tumor cell-conditioned medium, see Section 8, *infra*.

6.1.4. **ISOLATION OF MUTAGENIZED VECTORS**

In any of the above-described methods for isolating
super-infective, tumor-specific microorganisms, the "wild
35 type" parent microorganism can be subjected first to
mutagenesis before the microorganism is subjected to any
isolation or selection procedure of the present invention.

For example, bacteria are subjected to treatment with
nitrosoguanidine and ultraviolet B irradiation so that the
hereditary genetic material is modified resulting in the
altered expression of genes, both qualitatively and
5 quantitatively, in the microorganism. Other types of
chemical and high-energy mutagenesis are well known in the
art. For example, alkylating agents such as dimethyl
nitrosamine or ethyl methane sulfonate, or intercalating
agents, such as ethidium bromide. Other approaches include
10 transposon mutagenesis to introduce genetic flocks or fusions
of genes with new promoters. Any mutagen can be used in the
present invention to create mutant strains of microorganisms
which may then undergo any of the selection methods of the
present invention.
15   For an illustrative example of mutagenesis, see
Section 7.1, *infra*.

          6.2. **GENETIC MANIPULATION OF THE SELECTED VECTORS
               FOR DELIVERY OF GENES AND/OR GENE PRODUCTS TO
               THE TARGET SOLID TUMOR CELLS AS WELL AS FOR
20   ATTENUATION OF VIRULENCE**

          6.2.1.   **GENETIC MANIPULATION FOR DELIVERY OF
                    GENES AND/OR GENE PRODUCTS TO THE
                    TARGET SITE**
          After the selection processes described above in
25 which one obtains a super-infective, tumor-specific vector,
one can genetically engineer such vectors so that any desired
gene or gene product is delivered to a target site,
preferably the site of a solid tumor, more preferably, into
the tumor cell itself, the necrotic areas of the tumor or
30 into tumor-associated lymphocytes and macrophages.
Additionally, one can genetically alter naturally occurring
microorganisms which have a natural ability to infect tumor
cells and/or be tumor-cell specific. These vectors are
genetically engineered by a wide variety of methods known in
35 the art, for example, transformation or electroporation. In
a preferred embodiment of the present invention the vectors

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are engineered to deliver suicide genes to the target tumor
cells. These suicide genes include pro-drug converting
enzymes, such as *Herpes simplex* thymidine kinase (TK) and
bacterial cytosine deaminase (CD). TK phosphorylates the
5 non-toxic substrates acyclovir and ganciclovir, rendering
them toxic via their incorporation into genomic DNA. CD
converts the non-toxic 5-fluorocytosine (5-FC) into 5-
fluorouracil (5-FU), which is toxic via its incorporation
into RNA. Additional examples of pro-drug converting enzymes
10 encompassed by the present invention include cytochrome p450
NADPH oxidoreductase which acts upon mitomycin C and
porfiromycin (Murray et al., 1994, J. Pharmacol. Exp.
Therapeut. 270:645-649).

          Prodrug converting enzymes are being widely
15 employed for use in gene therapy of malignant cancers (Vile
and Hart, 1993, Cancer Res. 53:3860-3864; Moolten and Wells,
1990, J. Natl. Cancer Inst. 82:297-300; Wagner, et al., 1981,
Proc. Natl. Acad. Sci. USA 78:1441-1445; Mullen, 1994, Cancer
Res. 54:1503-1506; Huber et al., 1993, Cancer Res. 53:4619-
20 4625; Waldman et al., 1983, J. Biol. Chem. 258:11571-11575;
Mullen, et al., 1992, Proc. Natl. Acad. Sci. 89:33-37; Austin
and Huber, 1993, Mol. Pharmacol. 43:380-387). Table 2 is an
illustrative list of pro-drug converting enzymes (Bagshawe,
1995, Drug Dev. Res. 34:220-230).

25   Prodrug converting enzymes have been expressed in
several bacteria. The *Herpes simplex* virus has been
expressed in *E. coli* (Garapin, 1980, Proc. Natl. Acad. Sci.
USA 78:815-819; Waldman et al., 1983, J. Biol. Chem.
258:11571-11575). Similarly, Simula et al., 1993, Toxicology
30 82:3-20, expressed the prodrug converting enzyme cytochrome
p450 oxidoreductase in *Salmonella typhimurium* which confered
sensitivity to mitomycin.

35

TABLE 2

REPRESENTATIVE PRO-DRUG CONVERTING ENZYMES FOR USE IN VECTOR THERAPY

Enzyme	Pro-drug	Reference
Carboxypeptidase G2	benzoic acid mustards	Bashawe et al., 1988; Springer et al., 1990
	aniline mustards	Davies et al., 1994
	phenol mustards	Springer et al.
Beta-glucuronidase	p-hydroxyaniline mustard-glucuronide	Roffer et al., 1991
	epirubicin-glucuronide	Halsma et al., 1992
		Mitaku et al., 1994
Penicillin-V-amidase	adriamycin-N phenoxyaceryl	Kerr et al., 1990
Penicillin-G-amidase	N-(4'-hydroxyphenyl acetyl)-palytoxin doxorubicin	Bignami et al., 1992
	melphalan	Vrudhula et al., 1993
β-lactamase	nitrogen mustard-cephalosporin	Alexander et al., 1991
	β-phenylenediamine	
	vinblastine derivative-cephalosporin	
	cephalosporin mustard	Meyer et al., 1993
		Svensson et al, 1993
β-glucosidase	cyanophenylmethyl-β-D-gluco-pyranosiduronic acid	Rowlandson-Busza et al., 1991
Nitroreductase	5-(adaridin-1-yl-)2, 4-dinitrobenzamide	Knox et al., 1988; Somani and Wilman, 1994
Carboxypeptidase A	methotrexate-alanine	Haenseler et al., 1992

Bagshawe et al., 1988, Br. J. Cancer 58:700-703.

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Springer et al., 1990, J. Med. Chem. 33:677-681.

Davies et al., 1994, Ann. Oncol. 5 (Suppl 5):73(abstr).

Springer et al., 1994, A novel bisiodo-phenol mustard in antibody-directed enzyme pro-drug therapy (ADEPT). In: Programme of Eleventh Hammersmith Conference. Advances in the Application of Monoclonal Antibodies. London: Hammersmith Hospital (abstr).

5

Haisma et al., 1992(a), Cancer Immunol. Immunother. 34:343-348.

Roffler et al., 1991, Biochem. Pharmacol. 42:2062-2065.

Haisma et al., 1992(b), Br. J. Cancer 88:474-478.

Mitaku et al., 1994, Ann. Oncol. 5 (Suppl 5):76 (abstr).

10 Kerr et al., 1990, Cancer Immunol. Immunother. 31:202-206.

Bignami et al., 1992, Cancer Res. 52:5759-5764.

Vrudhula et al., 1993, J. Med. Chem. 38:919-923.

Alexander et al., 1991, Tetrahedron Lett. 32:3296-3272.

Meyer et al., 1993, Cancer Res. 53:3956-3963.

15 Svensson et al., 1992, Bioconj. Chem. 3:176-181.

Rowlandson-Busza et al., 1991, Cytotoxicity following specific activation of amygladin. In: Monoclonal Antibodies, Epenetos AA (ed), London: Chapman & Hall, pp. 179-183.

Knox et al., 1988, Biochem. Pharmacol. 41:4661-4669.

Somani et al., 1994, Ann. Oncol. 5 (Suppl 5):73 (abstr).

20 Haenseler, E., Esswein, A., Vitols, K.S., Montejano, V., Mueller et al., 1992, Biochemistry 31:214-220.

However, pro-drug converting enzymes such as the TK and CD enzymes, when synthesized in bacteria such as

25 *Salmonella* or *Escherichia coli*, are not normally secreted from the bacteria. Accordingly, the expression construct is designed such that the microorganism-produced gene products are secreted by the microorganism. Thus, TK or CD are able to generate phosphorylated acyclovir, ganciclovir, or 5-FU,

30 within the target tumor cell cytoplasm and interstitial spaces of the target tumor. Secretion of TK and CD is achieved by introducing into the expression construct a secretory signal sequence, for example, from the β-lactamase gene (Talmadge et al., 1980, Proc. Natl. Acad. Sci. USA

35 77:3369-3373).

Alternate signal sequences, in addition to β-lactamase, are also encompassed by the present invention.

Bacteria, for example, are known to have several means for
secretion into the periplasm and the outside media. The most
typical secretion sequences are N-terminal signal sequences
containing hydrophobic transmembrane spanning domains. These
5 sequences serve to guide the protein through the membrane and
are removed as- or after the protein crosses the membrane.
Prokaryotic and eukaryotic N-terminal signal sequences are
similar and it has been shown that eukaryotic N-terminal
signal sequences are capable of functioning as secretion
10 sequences in bacteria. In a preferred embodiment, the gene
encoding the enzyme β-lactamase (penicillinase) is used as
the source of the signal sequence. This signal sequence is a
well studied example of a bacterial enzyme which is secreted
both into the periplasm and into the external media.
15   Further, some bacterial proteins utilize a
different secretion signal which is located at the C-
terminus. The enteroinvasive *E. coli* hemolysin A *(hlyA)* is
the best studied member of this group. It has been shown
that the secretion signal is present in the last 60 amino
20 acids of that protein and that transfer of this domain to
other proteins can result in their direct secretion into the
media when the accessory proteins from the hemolysin operon
(hylC, A, B, & D) are present (Su et al., 1992, Microbial
Pathogen. 13:465-476). An illustrative list of secreted
25 proteins reviewed by Pugsley is presented in Table 3,
(Pugsley A. P., 1988, Protein secretion across the outer
membrane of gram-negative bacteria. In: Protein Transfer and
Organelle Biogenesis, R.C. Dand and P. W. Robbins (eds),
Academic Press, Inc., Harcourt Brace Jovanovich, Publishers,
30 San Diego, pp 607-652).

35

TABLE 3

SOURCES OF SECRETION SIGNALS FOR PRO-CONVERTING ENZYMES

Protein	Organism	Location in Transfected *E. coli* and type of signal	Ref. No.
Chitinase	*Serratia marcescens*	released into medium N-terminal signal	14
a-Hemolysin	*E. coli*	released into medium C-terminal signal	9
Heat labile enterotoxin I	various *E. coli* strains	Similar to cholera toxin 2 subunits (A&B); N-terminal signal in both; primarily in periplasm	12, 7, 29
Heat-stable enterotoxin I	various *E. coli* strains	N-terminal signal peptide; secreted into the media	11, 28
Heat-labile enterotoxin II	various *E. coli* strains	N-terminal signal peptide	17
Pullulanase	*Kelbsiella pneumoniae*	Release into the medium; N-terminal signal peptide	3, 27, 6
Serine protease	*S. marcencens*	Secreted into the medium; N-terminal signal peptide	31
Pectate lyase	*Erwinia chrysanthemi*	Mainly in the periplasm	15, 5
Pectate lyase	*E. carotovara*	Periplasm	18, 32
Protease	*E. chrysanthemi*	Secreted into the medium	30, 1
Aerolysin	*Aeromonas hydrophila*	Periplasm, N-terminal signal sequence (processed)	13
Phospholipase C	*Pseudomonas aeruginosa*	not secreted by *E. coli* N-terminal signal sequence	4, 19, 26
Exotoxin A	*P. aeruginosa*	not secreted by *E. coli*	10
Cholera toxin	*Vibrio cholerae*	Mainly periplasmic; 2 subunits	25, 20
Hemolysin	*V. cholerae*	Periplasm	21
DNase	*V. Cholerae*	Periplasm	22, 8
Thermostable Hemolysin	*V. parahaemolyticus*	Periplasm N-terminal signal peptide	24

TABLE 3

IgA protease	*Haemolphilis influenzae*	Periplasm	2
IgA protease	*Nisseria gonorrhoeae*	Secreted into the medium	16
Pertussis toxin	*Bordetella pertussis*	Periplasm; 5 subunits all with N-terminal signal peptides	23

1.   Barras et al., 1986, FEMS Microbiol. Lett. 34:343-348.

2.   Bricker et al., 1983, Proc. Natl. Acad. Sci. USA 80:2681-2685.

3.   Chapon et al., 1985, J. Bacteriol. 164:639-645.

4.   Coleman et al., 1983, J. Bacteriol. 153:909-915.

5.   Collmer et al., 1985, J. Bacteriol. 161:913-920.

6.   d'Enfert et al., 1987, EMBO J. 6:3531-3538.

7.   DaRas et al., 1980, Nature 288:499501.

8.   Focareta et al., 1985, FEMS Mcrobiol. Lett. 29:161-166.

9.   Goebel et al., 1984, Structure, function and regulation of the plasmid-encoded hemolysin determinant of *E. coli*. In Plasmids in Bacteria, D. R. Hehnski, S. N. Cohen, D. B. Cloewell, D. A. Jackson, and A. Hollaender (eds), pp. 791-805, Plenum, New York.

10.   Gray et al., 1984, Proc. Natl. Acad. Sci. USA 81:2645-2649.

11.   Guzmin-Verduzco et al., 1983, J. Bacteriol. 154:146-151.

12.   Hirst et al., 1984, Proc. Natl. Acad. Sci. USA 81:7752-7756.

13.   Howard et al., 1986, Mol. Gen. Genet. 204:289-295.

14.   Jones et al., 1986, EMBO J. 5:2377-2383.

15.   Keen et al., 1984, J. Bacteriol. 159:825-831.

16.   Koomey et al., 1982, Proc. Natl. Acad. Sci. USA 79:7881-7885.

17.   Lee et al., 1983, Infect. Immun. 42:264-268.

18.   Lei et al., 1985, Gene 35:63-70.

19.   Lory et al., 1983, Gene 22:95-101.

20.   Mekalanos et al., 1983, Nature 306:551-557.

21.   Mercuric et al., 1985, Mol. Gen. Genet. 200:472475.

22.   Newland et al., 1985, Infect. Immun. 47:691-696.

23.   Nicosia et al., 1986, Proc. Natl. Acad. Sci. USA 83:4631-4635.

24.   Nishibuchi et al., 1985, J. Bacteriol. 162:558-564.

25.   Pearson et al., 1982, Proc. Natl. Acad. Sci. USA 79:2976-2980.

26.   Pritchard et al., 1986, J. Bacteriol. 167:291-298.

27.   Pugsley, A. P., 1988, Protein secretion across the outer membrane of gram-
      negative bacteria. In: Protein Transfer and Organelle Biogenesis, R.C. Dand and P.
      W. Robbins (eds), Academic Press, Inc., Harcourt Brace Jovanovich, Publishers,
      San Diego, pp 607-652.

28.   So et al., 1980, Proc. Natl. Acad. Sci. USA 77:4011-4015.

29.   Spicer et al., 1982, J. Biol. Chem. 257:5716-5721.

30.   Wandersman C. (unpublished results cited in Pugsley, 1988).

31.   Yanigida et al., 1986, J. Bacteriol. 166:937-944.

32.   Zink et al., 1985, Appl. Environ. Microbiol. 49:714-717.

In another embodiment of the present invention, the
desired genes expressed from the expression constructs are
under the specific regulatory control of certain types of
promoters. These promoters may be either constitutive, in
which the genes are continually expressed, inducible, in
which the genes are expressed only upon the presence of an
inducer molecule(s) or cell-type specific control, in which
the genes, including but not limited to suicide genes, are
expressed only in certain cell types. Further, expression of
foreign genes including prodrug converting enzymes frequently
alters the phenotype of the bacteria. Therefore, it would be
an advantage to drive the expression of a prodrug converting
enzyme under exogenous control. This would allow
exploitation of the bacterial phenotypes such as tumor
targeting and amplification, after which time it would be
beneficial to express the prodrug enzyme. Inducible
promoters drive gene expression under specific conditions.
Furthermore, exogenously inducible promoters respond to
specific stimuli including chemical signals which can be
artificially introduced. It would be an advantage to drive
the expression of a prodrug enzyme using an exogenously
introduced agent which is approved for use in humans. The
"SOS" response of bacteria (Friedberg et al., In: DNA Repair
and Mutagenesis, pp. 407-455, Am. Soc. Microbiol. Press,
1995) is a response inducible by numerous agents including
chemotherapeutic alkylating agents such as mitomycin (Oda et

al., 1985, Mutation Research 147:219-229; Nakamura et al.,
1987, Mutation Res. 192:239-246; Shimda et al., 1994,
Carcinogenesis 15:2523-2529) which is approved for use in
humans. Promoter elements which belong to this group include

5 *umuC*, *sulA* and others (Shinagawa et al., 1983, Gene 23:167-
174; Schnarr et al., 1991, Biochemie 73:423-431). The *sulA*
promoter includes the ATG of the *sulA* gene and the following
27 nucleotides as well as 70 nucleotides upstream of the ATG
(Cole, 1983, Mol. Gen. Genet. 189:400-404). Therefore, it is

10 useful both in expressing foreign genes and in creating gene
fusions for sequences lacking initiating codons.

In one embodiment, for example, the expression of
the gene is controlled by a bacterial promoter which is
activated in specific target cells. In a preferred mode of

15 this embodiment, the bacterial promoter is activated
primarily in specific target cells. In another embodiment,
for example, the expression of the gene is controlled by a
bacterial promoter which is activated only in specific tumor
cells. An illustrative example of an expression construct

20 which expresses a gene under the control of a promoter with
the necessary secretion signal sequence is diagrammed in
Figure 1.

In a preferred embodiment of the present invention,
the expression of the gene is under the control of a promoter

25 which is active only in the target cell. Microorganism
promoters that are specifically or preferentially active in a
target tumor cell are isolated by a number of different
methods. For example, one method is using IVET (*in vivo*
expression technology) promoter trap procedure for isolating

30 specifically induced genes. This procedure is carried out by
taking, for example, a random pool of *Salmonella typhimurium*
DNA insertions generated by *Sau3A* restriction enzyme and
cloning the fragments into the promoter trap vector pIVET
(Slauch et al., 1994, Methods Enzymol. 235:481-492; Mahan et

35 al., 1993, Science 259:686-688). The cloning site is at the
position of the promoter for the *purA* gene which is required
for the synthesis of cyclic AMP. This representative pool is

transfected back into *Salmonella typhimurium* and an
integration event is induced which results in replacement of
the endogenous *purA* gene. The population of bacteria
carrying an integrated IVET plasmid is allowed to infect an
5 animal bearing a solid tumor of the cell type of choice and
after 24 hours bacteria are isolated from the tumor. Only
those bacteria that received a plasmid whose random piece of
Sau3A restricted DNA acts as a promoter within the tumor
cells is capable of surviving. In addition to controlling
10 the transcription of *purA*, the *Sau3A* restricted DNA promoter
also controls the expression of the β-galactosidase gene.
Two types of promoters are isolated which allow the survival
of bacteria within tumors, constitutive and regulated. The
constitutive promoter continues to control the positive
15 expression of both genes, inside and outside of the tumor.
Whereas the regulated promoter is no longer active in cells
other than the target cell.

        Another method for identifying promoters that are
active in tumors is to identify tumor-specifically induced
20 microbiological gene products using two dimensional gel
electrophoresis. For example, to determine which gene
products are specifically or preferentially expressed in
melanoma cells rather than macrophages, the method entails
three parallel infections which proceed in tandem: (1) 5×10^7
25 clonal microorganisms are allowed to infect 5×10^6 melanoma
cells, (2) 5×10^7 clonal microorganisms are allowed to infect
5×10^6 macrophages, and (3) 5×10^7 clonal microorganisms are
maintained in growth phase in LB broth. After a 30 minute
infection the cells are washed with DMEM with 10 μg/ml
30 gentamicin (for melanoma cells), RPMI 1640 with 10 μg/ml
gentamicin (for macrophages) and LB without gentamicin (for
free microorganisms). After two hours the cells are
pretreated with 50 mg/ml cyclohexamide to inhibit host cell
protein synthesis for 15 minutes. The cells are then washed
35 and placed in labeling medium (minus methionine) containing
75 μCi/ml ^{35}S-methionine for 30 minutes, followed by 1 hour in
normal medium. The cells are then harvested, denatured in 7M

urea buffer and subjected to isoelectrofocusing (IEF) followed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and analysis by autoradiography. Gene products specifically expressed in melanoma cells appear as
5 protein spots from microorganism-infected melanomas but not from microorganism-infected macrophages or from free microorganisms. The microorganismal genes that are specifically expressed are cloned from a λgt11 expression library using antiserum prepared from proteins derived from
10 preparative IEF and SDS-PAGE gels. Subsequent cloning from a cosmid library results in DNA fragments containing the promoter elements for the tumor-specific expressed gene product.

Yet another method for isolating promoters which
15 are specifically or preferentially activated in the target tumor cells is transposon mutagenesis. Transposon mutagenesis results in a pool of random mutants which can be tested for their ability to survive in epithelial cells but not in target tumor cells. Mutants are first tested for
20 their continued ability to persist in epithelial cells. Mutants no longer able to survive will be selected against. Surviving mutants are picked at random and placed in a numbered array using 96 well plates. The target tumor cells are grown in 96 well plates and individually infected with a
25 microorganism at a microorganism to host ratio of about 10:1 for 30 minutes, followed by washing and treatment with 10 μg/ml gentamicin. After 24 hours the plates are rinsed and stained with 0.4% trypan blue to determine the ratio of living cells(clear) to dead cells (blue) using a 96 well
30 plate reader. Microorganisms which are unable to survive within the target tumor cell are recovered from the original numbered plate. The genes are then cloned using the transposon as a genetic marker to isolate the DNA containing the tumor-specific expressed gene and its promoter.
35   The vectors which can express the various pro-drug or "suicide genes" when given to the host should not confer antibiotic resistance to the host and more importantly the

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bacteria should remain as sensitive to as many antibiotics as possible. Therefore, these vectors should not carry any antibiotic resistance markers. This can pose a problem in maintaining the expression vectors in the bacteria in absence

5 of selective pressure. However, the are a number of methods in which the vectors can be stably maintained without resorting to antibiotic resistance. For example, one such method is the construction of chromosomally integrated vectors expressing pro-drug converting enzymes or other

10 "suicide genes" as described by Donnenberg, 1991, Am. Soc. Microbiol., Annual Meeting, Abstract B-111, p.4; Donnenberg and Kaper, 1991, Infect. Immun. 59:4310-4317; and Ried and Collmer, 1987, Gene 57:239-246. Another method is the construction of stable episomal plasmids encoding "suicide

15 genes" or pro-drug converting enzymes using a balanced lethal system. Such balanced lethal systems are defined by the fact that the vector encodes for a function that compensates for a deficiency in the bacteria, such that the presence of the vector is essential for the survival of the bacteria. Such a

20 system is described by Galan et al., 1990, Gene 94:29-35. This system has the advantage over chromosomal integration in that the plasmids are multicopy and, therefore, achieve higher expression levels.

25   6.2.2.   **GENETIC MANIPULATION FOR ATTENUATION OF VIRULENCE**

          Many of the microorganisms encompassed by the present invention are causative agents of diseases in humans and animals. For example, sepsis from gram negative bacteria

30 is a serious problem because of the high mortality rate associated with the onset of septic shock (R.C. Bone, 1993, Clinical Microbiol. Revs. 6:57-68). Therefore, to allow the safe use of these vectors in both diagnostics and treatment of humans and animals, the microorganism vectors are

35 attenuated in their virulence for causing disease. In the present invention, attenuation, in addition to its traditional definition in which a microorganism or vector is

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modified so that the microorganism or vector is less
pathogenic, is intended to include also the modification of a
microorganism or vector so that a lower titer of that derived
microorganism or vector can be administered to a patient and
5 still achieve comparable results as if one had administered a
higher titer of the parental microorganism or vector. The
end result is to reduce the risk of toxic shock or other side
effects due to administration of the vector to the patient.
Such attenuated microorganisms are isolated in a number of
10 techniques. Such methods include use of antibiotic-sensitive
strains of microorganisms, mutagenesis of the microorganisms,
selection for tumor-specific, super-infective microorganism
mutants in culture or in tumor-bearing animals, selection for
microorganism mutants that lack virulence factors necessary
15 for survival in normal cells, including macrophages and
neutrophils, and construction of new strains of
microorganisms with altered cell wall lipopolysaccharides.
For example, in Section 6.1 et seq. where methods are
described for the isolation of super-infective, tumor-
20 specific vectors, these same methods are also methods for
isolating attenuated vectors; super-infective, tumor cell-
specific vectors are by definition attenuated. As the
vectors are highly specific and super-infective, the
difference between the number of infecting bacteria found at
25 the target tumor cell as compared to the non-cancerous
counterparts becomes larger and larger as the dilution of the
microorganism culture is increased such that lower titers of
microorganism vectors can be used with positive results.

Further, the microorganisms can be attenuated by
30 the deletion or disruption of DNA sequences which encode for
virulence factors which insure survival of the microorganisms
in the host cell, especially macrophages and neutrophils, by,
for example, homologous recombination techniques and chemical
or transposon mutagenesis. For example, a number of these
35 virulence factors have been identified in Salmonella. Many,
but not all, of these studied virulence factors are
associated with survival in macrophages such that these

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factors are specifically expressed within macrophages due to
stress, for example, acidification, or are used to induced
specific host cell responses, for example, macropinocytosis,
Fields et al., 1986, Proc. Natl. Acad. Sci. USA 83:5189-5193.
5   Table 4 is an illustrative list of *Salmonella* virulence
factors which, if deleted by homologous recombination
techniques or chemical or transposon mutagenesis, result in
attenuated *Salmonella*.

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TABLE 4

REPRESENTATIVE VIRULENCE FACTORS FOR *SALMONELLA TYPHIMURIUM* AND OTHER BACTERIA

Virulence Factor or Loci, Specific Stress Overcome or Stimulated Response	Reference
Acidification	Alpuche-Aranda et al., 1992
5'-adenosine monophosphate	Biochenko and Levashev, 1987
Cytolysin	Libbey et al., 1994
Defensin resistance loci	Fields et al., 1989
DNAK	Buchmeier and Hefferon, 1990
Fimbriae	Ernst et al., 1990
GroEL	Buchmeier and Hefferon, 1990
Induced Macropinocytosis	Alpuche Aranda, et al., 1994 Ginocchio et al., 1992 Jones et al., 1993
Inv loci	Betts and Finlay, 1992 Galon and Curtis III Ginocchio et al., 1992
Lipoprotein	Stone et al., 1992
LPS	Gianeiella et al., 1973 Stone et al., 1992
Lysosomal fusion inhibition	Ishibashi et al., 1992
Macropage survival loci	Fields et al., 1989
Oxidative stress (Sox; in *E. coli*)	Nunoshiba et al., 1993
PhoP and PhoQ	Behlau and Miller, 1993 Groisman et al., 1993 Miller et al., 1989

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TABLE 4

Pho activated genes (pag; *e.g.*, pagB and pagC)	Abshire and Neidhardt, 1993 Hefferon et al., 1992 Miller et al., 1992 Miller et al., 1989 Pulkkinen and Miller, 1991 Stone et al., 1992
PhoP and PhoQ regulated genes (*prg*)	Miller et al., 1989 Behlau and Miller, 1993; 1994
Porins	Tufano et al., 1988
Serum resistance peptide	Hackett et al., 1987
Virulence factors	Abshir and Neidhardt, 1993 Loos and Wassenaar, 1994 Mahan et al., 1995 Sansonetti, 1992
Virulence plasmid	Gulig and Curtiss, 1987 Rhen et al., 1993 Riikonen et al., 1992
spvB (virulence plasmid) traT (virulence plasmid) ty2	Fierer et al., 1993 Rhen and Sukupolvi, 1988 Elsinghorst et al., 1989

Abshiro et al., 1993, J. Bacteriol. 175:3734-3743

Alpuche-Aranda et al., 1992, Proc. Natl. Acad. Sci. USA 89:10079-83

Alpuche-Aranda et al., 1994, J. Exp. Med. 179:601-6088

Baumler et al., 1994, Infect. Immun. 62:1623-1630

Behlau et al., 1993, J. Bacteriol. 175:4475-4484

Belden et al., 1994, Infect. Immun. 62:5095-5101

Betts et al., 1992, Can. J. Microbiol. 38:852-7

Boichenko et al., 1987, Bull. Eksp. Biol. Med. 103:190-2

Boichenko et al., 1988, Zh. Mikrobiol. Epidemiol. Immunobiol. 7:9-11

Boichenko et al., 1985, Zh. Mkrobiol. Epidemiol. Immunobiol. 12:67-9

Bowe et al., 1994, Methods Enzymol. 236:509-26

Buchmeier et al., 1989, Infect. Immun. 57:1-7

5 Buchmeier et al., 1990, Science 248:730-732

Buchmeier et al., 1995, J. Cldn. Invest. 95:1047-53

Buchmeier et al., 1993, Mol. Microbiol. 7:933-936

Dragunsky et al., 1989, J. Biol. Stand. 17:353-60

10 Emoto et al., 1993, J. Immunol. 150:3411-3420

Ernst et al., 1990, Infect. Immun. 58:2014-2016

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10

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Yet another method for the attenuation of the
15 isolated vectors is to modify substituents of the
microorganism which are responsible for the toxicity of that
microorganism. For example, lipopolysaccharide (LPS) or
endotoxin is primarily responsible for the pathological
effects of bacterial sepsis. The component of LPS which
20 results in this response is lipid A (LA). Elimination or
mitigation of the toxic effects of LA results in an
attenuated bacteria since 1) the risk of septic shock in the
patient would be reduced and 2) higher levels of the
bacterial vector could be tolerated. *Rhodobacter*
25 *(Rhodopseudomonas) sphaeroides* and *Rhodobacter capsulatus*
each possess a monophosphoryl lipid A (MLA) which does not
elicit a septic shock response in experimental animals and,
further, is an endotoxin antagonist. Loppnow et al., 1990,
Infect. Immun. 58:3743-3750; Takayma et al., 1989, Infect.
30 Immun. 57:1336-1338.
Known similarities in lipid metabolism and genetic
organization of lipid metabolic genes between *Rhodobacter
sphaeroides* and other gram negative bacteria and the ability
of Rhodobacter genes to complement *E. coli* mutations (Benning
35 and Somerville, 1992(A), J. Bacteriol. 174:6479-6487;
1992(B), J. Bacteriol. 174:2352-2360; Carty et al., 1994,
FEMS Microbiol. Lett. 118(3):227-231) demonstrate that, for

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example, *Salmonella* and other bacteria can be genetically altered to produce MLA, thereby reducing its potential of inducing septic shock. A preferred embodiment of the present invention is a *Salmonella* spp. strain that expresses MLA

5 rather than LA and also expresses *HSV* TK under the control of a tumor-specific promoter.

As an illustrative example, the generation of MLA producing *Escherichia coli* or *Salmonella typhimurium* entails constructing a DNA gene library composed of 10 kB fragments

10 from *Rhodobacter sphaeroides* which is generated in λgt11 or pUC19 plasmids and transfected into *E. coli*. Clones which produce MLA are positively selected by using an antibody screening methodology to detect MLA, such as ELISA. In another example one generates a cosmid library composed of 40

15 kB DNA fragments from *Rhodobacter sphaeroides* in pSuperCos which is then transfected into *Salmonella typhimurium*. Clones which produce MLA are positively selected by using an antibody screening methodology to detect MLA, such as ELISA.

Yet another example for altering the LPS of

20 *Salmonella* involves the introduction of mutations in the LPS biosynthetic pathway. Several enzymatic steps in LPS biosynthesis and the genetic loci controlling them in both *E. coli* and *Salmonella typhimurium* have been identified (Raetz, 1993, J. Bacteriol. 175:5745-5753 and references

25 therein). Several mutant strains of *Salmonella typhimurium* and *E. coli* have been isolated with genetic and enzymatic lesions in the LPS pathway. One such mutant, *firA* is a mutation within the gene that encodes the enzyme UDP-3-O(R-30 hydroxymyristoyl)-glycocyamine N-acyltransferase, that

30 regulates the third step in endotoxin biosynthesis (Kelley et al., 1993, J. Biol. Chem. 268:19866-19874). *Salmonella typhimurium* and *E. coli* strains bearing this type of mutation produce a lipid A that differs from wild type lipid A in that it contains a seventh fatty acid, a hexadecanoic acid (Roy

35 and Coleman, 1994, J. Bacteriol. 176:1639-1646). Roy and Coleman demonstrated that in addition to blocking the third step in endotoxin biosynthesis, the *firA*⁻ mutation also

- 50 -

decreases enzymatic activity of lipid A 4' kinase that
regulates the sixth step of lipid A biosynthesis.

   Once the strain has been attenuated by any of the
methods known in the art, the stability of the attenuated
5 phenotype is important such that the strains do not revert to
a more virulent phenotype during the course of treatment of a
patient. Such stability can be obtained, for example, by
providing that the virulence gene is disrupted by deletion or
other non-reverting mutations on the chromosomal level rather
10 than epistatically or that the "suicide gene" is stably
integrated into the bacterial chromosome.

   Another method of insuring the attenuated phenotype
is to engineer the bacteria such that it is attenuated in
more than one manner, e.g., a mutation in the pathway for
15 lipid A production, such as the firA⁻ mutation (Hirvas et al.,
1991, EMBO J. 10:1017-1023) and one or more mutations to
auxotrophy for one or more nutrients or metabolites, such as
uracil biosynthesis, purine biosynthesis, and arginine
biosynthesis as described by Bochner, 1980, J. Bacteriol.
20 143:926-933. In a more prefered embodiment of the invention,
the bacterial vector which selectively targets tumors and
expresses a pro-drug converting enzyme is auxotrophic for
uracil, aromatic amino acids, isoleucine and valine and
synthesizes an altered lipid A.
25

   6.3. *IN VITRO* CANCER DIAGNOSTICS AND *IN VIVO*
         TREATMENT OF SOLID TUMORS USING ISOLATED
         VECTORS AND OTHER VECTORS

   6.3.1.   *IN VITRO* DIAGNOSTICS
30
   An embodiment of the present invention is to
provide methods for use of the vectors of the present
invention in *in vitro* diagnostic assays and diagnostic kits
for the detection of solid tumor cancers, including but not
limited to melanoma. Also, the kits may comprise tumor-
35 specific non-attenuated vectors. The *in vitro* diagnostic
assays and kits are based on the enhanced specificity towards

a cancerous cell rather than its non-cancerous counterpart
cell of a vector. For example, and not by way of limitation,
a putative solid tumor is biopsied from a patient. The tumor
biopsy is minced and digested to a suspension of single

5 cells. Aliquots of the suspension and a non-cancerous
counterpart or control cell are cultured and infected with a
tumor-specific vector according to the present invention.

         After an incubation period, the number of tumor-
specific microorganisms which attached to and/or infected the

10 biopsied cells as compared to the non-cancerous counterpart
or control cells is determined by any method known to those
skilled in the art. A higher number of vectors found
associated with the target cell as compared to the non-
cancerous counterpart or control cells indicates that the

15 target cell is cancerous, for example, about 5-10 times as
many vectors will infect a tumor cell compared to a non-
cancerous control cell. A non-cancerous counterpart or
control cell is the normal cell from which the tumor cell is
derived, for example, for melanoma cells the non-cancerous

20 counterpart or control cell is melanocyte cells, for colon
cancer the counterpart cell is colon epithelial cells. In
one embodiment the ratio is determined as the number of
vectors/target cell. In another embodiment, after infection,
the cells are fixed and treated with a stain or antibody

25 which recognizes DNA so that the vector DNA present in the
target cell cytoplasm is visualized. The presence of DNA in
the target cell cytoplasm indicates that the biopsied target
cells are cancerous. In one embodiment of the present
invention the diagnostic method comprises exposing a sample

30 of cells which are suspected of being cancer cells to a
tumor-specific vector or microorganism. The method also
comprises exposing a sample of non-cancerous counterpart
cells to the tumor-specific vector or microorganism as a
comparative control. After incubating for a time period in

35 which the microorganisms can attach to and/or infect cancer
cells, the infectivity of the microorganism or vector for the

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cells suspected of being cancerous and the non-cancerous
counterpart control cells can be compared.

     The diagnostic kits of the present invention
comprise an effective amount of a tumor-specific vectors.
5 The kits can further comprise an appropriate amount of non-
cancerous control cells. The vector and/or cells may be
supplied either frozen, lyophilized or growing on solid or in
liquid medium. The diagnostic kits can further comprise
inert ingredients and other kit components such as vials,
10 packaging components and the like, which are well known to
those skilled in the art.

     In certain embodiments, the vectors useful for the
methods of diagnosis of the present invention can further
comprise tumor-specific, attenuated or non-attenuated
15 vectors. In other embodiments, the kits of the present
invention can comprise tumor-specific, attenuated or non-
attenuated vectors.

     For illustrative examples of in vitro diagnostics
of solid tumor cancers, including but not limited to
20 melanoma, see Sections 22, 25 and 26.

6.3.2. *IN VIVO* TREATMENT OF SOLID TUMORS

     The vectors for use in *in vivo* cancer treatment are
a subset of the vectors of the present invention. The
25 vectors for *in vivo* treatment have been attenuated such that,
when administered to a host, the vector has been made less
toxic to the host and easier to eradicate from the host's
system. In a preferred embodiment, the vectors are super-
infective, attenuated and specific for a target tumor cell.
30 In a more preferred embodiment, the vectors are also
sensitive to a broad range of antibiotics.

     In addition, the isolated vectors can encode
"suicide genes", such as pro-drug converting enzymes or other
genes, which are expressed and secreted by the vector in or
35 near the target tumor. The gene can be under the control of
either constitutive, inducible or cell-type specific
promoters. In a preferred embodiment, a suicide gene is

expressed and secreted only when a vector has invaded the
cytoplasm of the target tumor cell, thereby limiting the
effects due to expression of the suicide gene to the target
site of the tumor.

5   In a preferred embodiment, the vector, administered
to the host, expresses the *HSV* TK gene. Upon concurrent
expression of the TK gene and administration of ganciclovir
to the host, the ganciclovir is phosphorylated in the
periplasm of the microorganism which is freely permeable to
10 nucleotide triphosphates. The phosphorylated ganciclovir, a
toxic false DNA precursor, readily passes out of the
periplasm of the microorganism and into the cytoplasm and
nucleus of the host cell where it incorporates into host cell
DNA, thereby causing the death of the host cell.

15   Another embodiment of the present invention is to
provide methods of treatment of solid tumor cancers with
isolated attenuated vectors of the present invention. For
example, a patient is diagnosed with a solid tumor cancer by
any method known in the art, including the *in vitro*
20 diagnostic methods of the present invention. The vector used
in the treatment may already be isolated using the methods of
the present invention with target cell lines or using model
tumors in mice of the target tissue. In another embodiment,
the biopsied tumor cells are used in the selection assay for
25 isolating a vector which is super-infective and tumor-
specific for the tumor of the patient. In a preferred
embodiment the vector is genetically modified, for example,
to lack virulence factors, express a suicide gene or both as
described in Section 6.2.2. In addition, the isolated vector
30 is analyzed for sensitivity to antibiotics to insure the
eradication of the vector from the patient's body after
successful treatment or if the patient experiences
complications due to the administration of the isolated
vector.

35   When administered to a patient, *e.g.*, an animal for
veterinary use or to a human for clinical use, the vectors
can be used alone or may be combined with any physiological

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carrier such as water, an aqueous solution, normal saline, or
other physiologically acceptable excipient. In general, the
dosage would range from about 1 to 1x10^8 c.f.u./kg, preferably
about 1 to 1x10^2/kg.

5   The vectors of the present invention can be
administered by a number of routes, including but not limited
to: orally, topically, injection including, but limited to
intravenously, intraperitoneally, subcutaneously,
intramuscularly, intratumorally, *i.e.*, direct injection into
10 the tumor, etc.

             The following series of examples are presented by
way of illustration and not by way of limitation on the scope
of the invention.

15   7.   **EXAMPLE: ISOLATION OF SUPER-INFECTIVE, TUMOR-
             SPECIFIC *SALMONELLA TYPHIMURIUM IN VITRO***

             7.1. **MUTAGENESIS BEFORE ISOLATION OF SUPER-
                   INFECTIVE, TUMOR-SPECIFIC CLONES**

             A culture of *Salmonella typhimurium* strain #14028
20 was grown exponentially at 37°C in minimal medium 56 plus
glycerol (0.5%) to OD^{600}= 0.3, then chilled on ice. An
aliquot was removed so that the culture could be titered for
colony forming units (c.f.u.) on LB agar plates. The culture
was washed and resuspended in Na citrate (0.1M, pH 5.5),
25 incubated with fresh nitrosoguanidine (NG, 50μg/ml, 20
minutes, 37°C), washed once by centrifugation, resuspended in
medium 56, chilled, and again an aliquot was removed so that
the culture could be titered for c.f.u. on LB agar plates.
Another aliquot of the NG treated bacteria was diluted (1:5)
30 into LB broth and grown to stationary phase for storage
frozen at -80°C in 12% glycerol.

             The remaining bacteria were irradiated with
ultraviolet light, dose = 50J/m2, λ= 254nm). An aliquot was
removed and the cells were then titered for c.f.u. on LB agar
35 plates, with another aliquot diluted 1:4 into LB broth, grown
to stationary phase, and stored frozen at -80°C in 12%
glycerol.

The mutagenesis procedures produced an increase in the number of mutations in the strain by four criteria: 1) decreased survival of the bacteria following mutagenesis (nitrosoguanidine=6-fold; ultraviolet B irradiation=400-fold); 2) increased frequency of auxotrophic (nutritional requiring) mutants to (2%); 3) increased frequency of maltose⁻ mutants to (2%); 4) increased frequency of galactose⁻ mutants to (0.5%).

7.2. ISOLATION OF SUPER-INFECTIVE *SALMONELLA TYPHIMURIUM* CLONES #70 AND #71 SPECIFIC FOR CANCER CELLS

A population of *Salmonella typhimurium* wild type strain #14028 was mutagenized as described in Section 7.1 with nitrosoguanidine and UV irradiation. Briefly, the bacteria were grown exponentially at 37°C in minimal medium 56 plus glycerol to $OD^{600}=0.3$, chilled on ice, washed, resuspended in Na citrate with 50μg/ml nitrosoguanidine and incubated for 20 minutes at 37°C. The bacteria were washed once by centrifugation and resuspended in medium 56. The bacteria were then irradiated with UV light at a dose of $50J/m^2$, $\lambda=254nm$.

Prior to infection by *Salmonella*, human M2 melanoma cells were inoculated into Coming Tissue Culture flasks (25cm²) at approximately 2×10^5 cells/flask in 4 ml DMEM cell culture medium containing penicillin (100 units/ml), and streptomycin (100μg/ml), and incubated overnight in a 37°C, gassed (5%CO2), humidified incubator. The next day the cells were rinsed twice with prewarmed Dulbecco's Minimal Essential medium supplemented with 10% fetal bovine serum (DMEM/FBS) and no antibiotics.

The mutated population of *Salmonella typhimurium* was cultured on LB agar overnight at 37°C or in a liquid culture. The following day the bacteria were transferred with a platinum wire loop to LB broth or to DMEM/FBS, adjusted in concentration to $OD^{600}=0.1$ (approximately 2×10^8 c.f.u./ml), and subjected to further growth at 37°C on a

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rotator. Following growth to the desired population density
(monitored at an optical density of 600nm) the bacteria were
diluted to a concentration of 10^6 c.f.u./ml in DMEM/FBS, and
incubated at 37°C an additional 20 minutes.

5   The mutagenized bacterial population was subjected
to a single cycle of infection into- and isolation from human
M2 melanoma cells in culture. Portions of the mutagenized
population were grown clonally on agar and 20 clones of
Salmonella typhimurium were separately isolated and tested

10 for their individual infectivity toward human M2 melanoma
cells. The bacteria were added to animal cell cultures in 25
cm^2 Coming Tissue Culture flasks at 4ml/flask, and incubated
with the animal cells in a gassed (5%CO^2/95% air), humidified
incubator at 37°C. After a 15 minute incubation with the

15 animal cells the bacteria-containing medium was poured off
and the cultures were rinsed gently with warmed DMEM/FBS
(4 ml) containing gentamicin sulfate (20µg/ml), an antibiotic
that kills extracellular but not intracellular bacteria. The
gentamicin sulfate-containing medium was poured off, fresh

20 DMEM/FBS/gentamicin sulfate medium was added, and the cells
were incubated for 60 minutes at 37°C. Following the 60
minute incubation with gentamicin sulfate, the medium was
poured off, the flasks were rinsed 1x with DMEM/FBS (without
gentamicin sulfate), and 1 mM EDTA or an EDTA/trypsin

25 solution (Sigma Chemicals, 1x) in Ca^{++}/Mg^{++} free physiological
saline (4 ml) was added. After incubating with EDTA or
EDTA/trypsin for 20 minutes at 37°C, the flasks were shaken
to suspend the animal cells, and aliquots were removed for
quantitation. Animal cells were quantitated in a Coulter

30 Counter (Coulter Electronics, Inc.) and bacteria were
quantitated by plating aliquots on LB agar, incubating at
37°C, and counting colonies. Quantitation was expressed as
the number of infecting (gentamicin resistant) bacteria/10^6
animal cells.

35   Two clones, "70" and "71", were found to be super-
infective of melanoma cells, with infection capacities 5-10-
fold greater than the mutagenized wild type strain (data not

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shown). Clones 70 and 71 were also assessed for their
relative specificity of the following human cells in culture:
M2 melanoma cells and normal human melanocytes; "CaCo" colon
cancer cells and normal human colon epithelium #1790 as
5 depicted in Table 4(A).

TABLE 4(A)

SPECIFIC INVASION OF S. TYPHIMURIUM INTO MELANOMA VS MELANOCYTES AND COLON CANCER VS COLON EPITHELIUM IN CELL
10 CULTURE: CLONES "70" AND "71"+

Human	Infecting Salmonella/10^6		human cells:	
Cell Line	Clone 70	(ratio)*	Clone 71	(ratio)*
normal melanocytes	$1.4 \pm 0.2 \times 10^6$		$1.2 \pm 0.3 \times 10^6$	
M2 melanoma	$7.3 \pm 2.0 \times 10^6$	(5.2)	$5.7 \pm 0.7 \times 10^6$	(4.8)
colon epithelium (#1790)	$1.5 \pm 0.1 \times 10^6$		$0.8 \pm 0.2 \times 10^6$	
colon carcinoma (CaCo)	$7.2 \pm 2.0 \times 10^6$	(4.8)	$2.3 \pm 0.3 \times 10^6$	(2.9)

25 *cancer cell:normal counterpart cell
+Results represent averages ± SD for triplicate infections.

The bacterial clones #70 and #71 showed strong invasion
preference for melanoma and colon cancer cells over that for
normal melanocytes and normal colon epithelial cells.
30

7.3. ISOLATION OF SALMONELLA TYPHIMURIUM SUPER-INFECTIVE CLONE #72 BY CYCLING IN IN VITRO CELL CULTURE

Salmonella wild type strain #14028 was mutagenized
with nitrosoguanidine and ultraviolet B irradiation as
35 described in Section 7.1. A starting population of 5 x 10^8
mutagenized bacteria was grown to $OD^{600}=.450$, diluted in

DMEM/FBS to a concentration of 5 x 10^7 c.f.u./ml, and allowed
to infect human M2 melanoma cells for 15 minutes. Infecting
bacteria were isolated from the melanoma cells, and again
allowed to infect fresh, uninfected populations of melanoma
5   cells. The 2nd round of infecting bacteria were again
isolated and subjected to further cycles of infection into,
and isolation from, human M2 melanoma cells. After the
completion of 4 such cycles, the population of melanoma-
cycled bacteria which is designated 14028^{POP-1} was then plated
10 on agar and 100 individual clones were picked and tested for
their ability, compared to wild type bacteria, to infect M2
melanoma cells. The results of the selection process on
14028^{POP-1} and selected population sub-clones are detailed in
Table 5.

15   Additionally, an aliquot of 14028^{POP-1} was subjected
to two further cyclings in M2 melanoma cells. This 6X-cycled
population was then subjected to 7 cycles of negative
selection against normal human melanocytes. The 6X-cycled
population was added to a culture of normal human melanocytes
20 and incubated for 15 minutes. The supernatant was collected
and was then added back to a fresh culture of normal human
melanocytes. This negative selection procedure was carried
out 7 times. This 6X-7X cycled population was again added to
M2 melanoma cells, allowed to infect the melanoma cells for
25 15 minutes, and the bacteria were then collected from the M2
cells.   This 6X-7X-1X cycled population was designated
14028^{POP-2}.

           The mixed population of 4 times cycled *Salmonella
typhimurium*, designated 14028^{POP-1}, showed a 3-fold increased
30 infectivity of melanoma cells over that of the starting
mutagenized population of wild type bacteria. Of the 100
clones isolated from population 14028^{POP-1} of *Salmonella*, two
clones, #6 and #72, were found to be significantly super-
infective of melanoma cells. The remaining bacterial clones
35 showed infectivity that was similar to or below that of the
wild type strain. In the experiment presented in Table 5,
clone #6 was about 25-fold, and clone #72 was about 55-fold

more infective than the mutagenized wild type strain during a
15 minute infection period. *Escherichia coli*, strain K-12,
#CSH 101, was at least two orders of magnitude less infective
than wild type *Salmonella typhimurium*, thus, demonstrating
5 the natural ability of *S. typhimurium* to infect certain
animal cells.

TABLE 5

INFECTION OF M2 HUMAN MELANOMA CELLS WITH VARIOUS ISOLATED, *SALMONELLA TYPHIMURIUM* POPULATIONS IN CULTURE[+]

Salmonella Strain	Infecting Bacteria/10^6	melanoma cells (%wild type)
Wild type *S. typhimurium* #14028 (mutagenized)	$3.8 \pm 3.0 \times 10^4$	100
#14028[pop-1]	$1.1 \pm 0.4 \times 10^5$	290
Clone #6	$8.6 \pm 1.0 \times 10^6$	2260
Clone #72	$2.1 \pm 0.2 \times 10^6$	5500
E. coli K-12	$< 10^2$	<1

[+]Results represent averages $\pm$ SD for triplicate infections.

Over several such experiments shown in Table 5, the
25 infectivity of clone #72 toward melanoma cells varied from 5-
to 90-fold over that of the wild type strain. This variation
seemed to depend on the bacterial growth density prior to
infection of melanoma cells. Therefore, the effect of
population density on relative infectivity between wild type
30 and clone #72 was determined.

Wild type *S. typhimurium* and super-infective clone
#72 were grown as a lawn on LB agar plates. Portions of the
cultures were removed with a platinum loop and inoculated
into LB broth at a concentration of approximately 2×10^8
35 c.f.u./ml ($OD^{600}=0.1$). The cultures were then placed on a
rotator at 37°C and optical densities were monitored as a
function of population density. At the optical densities

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indicated, aliquots of bacteria were removed, diluted in
melanoma growth medium (DMEM/10% FBS) to a density of 1×10^6
c.f.u./ml, and allowed to infect human M2 melanoma cells.
Infectivity assays were carried out as described. The
5 results are shown in Table 6.

TABLE 6
INFECTIVITY OF WILD TYPE *S. TYPHIMURIUM* AND SUPER-INFECTIVE CLONE #72 TOWARDS HUMAN MELANOMA CELLS: EFFECT OF BACTERIAL POPULATION DENSITY+

Optical Density: *Salmonella*/10^6 melanoma		Infectivity ratio:
(600nm)	Clone #72	Wild type	(Clone 72:wild type)
0.200	-0-	-0-	(no infectivity)
0.300	9.0×10^3	-0-	(infinite)
0.400	5.0×10^4	-0-	(infinite)
0.500	4.5×10^5	5.0×10^3	90:1
0.600	1.2×10^6	3.7×10^4	32:1
0.700	2.3×10^6	2.5×10^5	9:1
0.800	3.2×10^6	5.6×10^5	6:1
0.900	3.6×10^6	7.3×10^5	5:1

+Results represent averages of duplicate experiments. Variations between duplicates
were $< \pm 15\%$.
25

The results demonstrate that infectivity of both
bacterial strains was highly dependent on bacterial
population density prior to infection, however, clone #72 was
proportionately more infective than the wild type strain at
30 low population densities.
The results shown in Tables 5 and 6 were also
confirmed by phase and light microscopy which revealed super-
infectivity of a 10x melanoma-cycled population of *Salmonella*
typhimurium designated "M10" as shown in Figures 2 and 3. It
35 was also found that wild type strain 14028 and clone 72
infected human M2 melanoma cells equally well when grown

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under anaerobic conditions prior to infection. However, when the strains are grown under aerobic conditions, strain 14028 was strongly suppressed in infectivity, whereas clone 72 remained induced. Thus, clone 72 was infective under either

5 anaerobic or aerobic growth conditions and superinfective compared to wild type under aerobic growth conditions.

7.4. PREFERENTIAL SELECTIVITY OF *S. TYPHIMURIUM* FOR CANCER CELLS: WILD TYPE STRAIN VS. SUPER-INFECTIVE CLONE #72

10 Super-infective *Salmonella typhimurium* clone #72 isolated in Section 7.3 was compared to the non-mutagenized wild type strain #14028 for relative infectivity of human M2 melanoma cells, normal human melanocytes, colon cancer cells and normal colon epithelium. The results are shown in Table

15 7.

20

25

30

35

TABLE 7

TUMOR SPECIFICITY OF WILD TYPE *S. TYPHIMURIUM* AND SUPER-INFECTIVE CLONE #72 TOWARD VARIOUS NORMAL AND CANCEROUS CELLS IN CULTURE[+]

Animal	Infecting *Salmonella*/ 10^6 animal cells			
Cell Line	wild type	(ratio)[*]	Clone #72	(ratio)[*]
normal melanocyte (foreskin, human)	$1.2 \pm 0.7 \times 10^4$		$2.7 \pm 0.4 \times 10^5$	
M2 human melanoma	$2.5 \pm 0.6 \times 10^4$	(2.1)	$1.7 \pm 1.1 \times 10^6$	(6.3)
normal colon epithelium (1790, human)	$6.6 \pm 0.8 \times 10^3$		$5.2 \pm 3.0 \times 10^5$	
colon cancer (HTB 39, human)	$3.0 \pm 2.0 \times 10^4$	(4.6)	$9.5 \pm 3.0 \times 10^5$	(1.8)
"normal" fibroblast (3T3, mouse)	$1.8 \pm 1.5 \times 10^4$		$5.5 \pm 1.4 \times 10^5$	
transformed macrophage (J774, mouse)	$2.4 \pm 0.6 \times 10^4$	(1.3)	$4.6 \pm 0.8 \times 10^6$	(8.4)

[+]Results represent averages ± SD for triplicate infections.
[*]cancer cell:normal counterpart cell

Each of the two bacterial strains showed invasion preference for human cancer cells over normal cells. Clone #72 was super-infective in all cases when compared to the wild type strain. Further, clone #72 showed a significantly higher degree of invasion specificity for human melanoma cells over normal melanocytes than the wild type strain did.

7.5. INFECTIVITY OF *SALMONELLA TYPHIMURIUM* WILD
TYPE STRAIN 14028 AND SUPERINFECTIVE CLONE 72
TOWARD VARIOUS HUMAN CARCINOMAS IN CULTURE

In another series of experiments, the relative
infectivities of clone 72 and wild type strain 14028, toward
a variety of human carcinomas growing in culture, was
determined. The experimental protocol used is described in
Section 7.2. Results are presented in Table 7(A).

TABLE 7(A)

INFECTIVITY OF *SALMONELLA TYPHIMURIUM* WILD TYPE AND SUPERINFECTIVE CLONE 72 TOWARD VARIOUS HUMAN CARCINOMAS IN CULTURE

Salmonella/10^6 Human Cells

Cell Line	Origin of Primary Tumor	Wildtype: 14028	Clone # 72	Ratio 72:14028
M2	melanoma	$4.0\pm3.8 \times 10^4$	$4.2\pm3.5 \times 10^5$	11:1
HTB57	lung	$2.8\pm1.3 \times 10^3$	$4.5\pm2.1 \times 10^4$	16:1
HTB183	lung	$1.0\pm0.3 \times 10^5$	$4.1\pm1.8 \times 10^5$	4:1
HTB54	lung	$2.1\pm0.7 \times 10^4$	$1.7\pm0.2 \times 10^5$	8:1
A549	lung	$3.7\pm5.6 \times 10^4$	$4.5\pm4.9 \times 10^5$	12:1
CRL1740	prostate	$2.3\pm0.4 \times 10^5$	$1.8\pm0.2 \times 10^6$	8:1
CRL1611	kidney	$3.2\pm1.2 \times 10^5$	$1.8\pm0.3 \times 10^5$	6:1
HTB52	liver	$1.8\pm0.3 \times 10^5$	$2.6\pm0.8 \times 10^5$	1.4:1
MCF7	breast	$7.3\pm2.6 \times 10^4$	$3.6\pm0.9 \times 10^5$	5:1

Results represent averages ± SD for n = 3-9 separate infections.

Both the wild type strain 14028 and clone #72 were
able to infect each of the human cancer cells tested in
culture. In all cases, clone 72 was superinfective compared
to the wild type strain.

However, human lung line HTB57 was significantly
less receptive to *Salmonella typhimurium* infectivity when
compared to other cancer cell lines tested. In yet another

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series of experiments, the human lung line HTB57 was
implanted into mice.   In 10 of 10 *nu/nu* mice implanted with 1
x 10^7 HTB57 cells, no tumor "takes" were observed, even after
several months.   Whether or not these cells were receptive to
5 *Salmonella* infection when grown as tumors was not determined.

7.6. DISCUSSION

In summary, the results demonstrate the following:
a) infectivity of *S. typhimurium* is dependent upon population
10 density of the bacteria and b) super-infective clone #72
differs from the wild type strain in its increased
infectivity of melanoma cells at all bacterial population
densities and especially at low population densities under
aerobic growth conditions.   The ability to infect at low
15 bacterial population densities is an advantage in the use of
clone #72 as a tumor-specific vector, since it would allow
for a lower c.f.u. of bacteria inoculated into the cancer
patient, thus reducing the risk of septic shock in the
patient.   Additionally, the results demonstrate methods for
20 the isolation of super-infective, tumor-specific mutants of
S. typhimurium.   Such mutants are represented by clones #6,
#70, #71 and #72 that were isolated via enrichment procedures
for melanoma infectivity by the bacteria.   The results
further demonstrate that wild type *S. typhimurium* exhibits
25 specificity for human cancer cells over normal human cells in
culture.   Further, although clone 72 was originally selected
for superinfectivity toward human melanoma cell line M2, it
was additionally found to be superinfective toward human
colon cancer cells and transformed mouse macrophages, when
30 compared to the wild type strain 14028 (*see* Table 7).   The
expression of super-infectivity and tumor-specificity of
isolated mutant clones represent attenuation of the bacteria
and present distinct advantages for the use of such
Salmonella clones as tumor-specific vectors in the diagnosis
35 and therapy of human cancer.

8.   **EXAMPLE: SELECTION FOR *SALMONELLA TYPHIMURIUM*
      MUTANTS WITH CHEMOTACTIC ABILITIES TOWARD MELANOMA
      SECRETORY PRODUCTS *IN VITRO***

The melanoma cells were an artificially-produced
hybrid line isolated from a polyethylene glycol induced
fusion between Cloudman S91 mouse melanoma cells and
peritoneal macrophages from a DBA/2J mouse. The hybrid cell
line used herein was termed Cloudman S91 melanoma/macrophage
hybrid #48. The hybrid cell line formed rapidly growing
metastasizing tumors in DBA/2J mice, Pawelek et al., 1995, J.
Invest. Dermatol. 104:605. 5 x 10⁶ Cloudman S91
melanoma/macrophage hybrid #48 cells were cultured at 30°C in
a gassed, humidified incubator in 75cm² culture flasks in
DMEM/FBS culture medium containing 10% fetal bovine serum and
no antibiotics. Control flasks containing DMEM/FBS but no
melanoma cells were incubated in parallel. After 72 hours,
the media were removed, aseptically filtered through 0.45μ
filters, and stored at 4°C.

Salmonella typhimurium super-infective clone #72
described above was subjected to mutagenesis with
nitrosoguanidine and UV. The mutagenesis procedures produced
an increase in the number of mutations in Clone #72 similar
to that shown earlier when the wild type strain #14028 was
mutagenized. This mutagenized population of clone 72
("72^mut") was further used to select for mutants with enhanced
chemotactic abilities toward melanoma cell secretory
products, *i.e.*, melanoma-conditioned culture media.

Procedures for loading capillary tubes with
potential chemotactic attractants were modified from Adler
(Adler, 1973, J. General Microbiology 74:77-91). Control and
melanoma-conditioned culture media, described above, were
loaded into 2λ capillary tubes ("Microcaps", Drummond
Scientific Co.) as described by Adler. The capillaries were
handled with forceps. One end was sealed in a flame; the
capillary was then quickly passed several times through the
flame and immediately plunged open end down into a 10 ml
beaker containing 1 ml control or melanoma conditioned

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culture medium.   As the capillary cooled (about 10 minutes),
liquid was drawn in about 1 cm.

      Salmonella typhimurium, growing at 37°C in LB were
collected by centrifugation and resuspended in control
5 DMEM/FBS culture medium containing a concentration of 10^8
c.f.u./ml.   Aliquots (200 μl, 2 x 10^7 c.f.u.) were pipetted
into 1.5 ml microfuge tubes.   Loaded capillary tubes
(described above) were then inserted open end down into the
Beckman microfuge tubes containing the Salmonella
10 typhimurium, and the assay was begun by incubating at 37°C.
After 30 to 60 minutes, the capillary tubes were removed with
forceps, the sealed ends were broken off with wire cutters,
and the capillaries were transferred to 15 ml conical
centrifuge tubes containing 3 ml LB broth.   It was important
15 that the upper tips of capillary tubes were covered with LB
broth in order to assure quantitative recovery of the
bacteria via the centrifugation step described as follows.
The capillaries within the centrifuge tubes were then
centrifuged (1000x g for 4 minutes) to force the bacteria out
20 of the capillaries.   The bacteria were resuspended by
vortexing, and aliquots were spread onto LB agar plates for
quantitation.   Significant increases in the number of
bacteria entering the capillaries containing melanoma-
conditioned media compared to control-conditioned media
25 indicated a chemotactic response of the bacteria to melanoma-
secreted products.

      Aliquots of mutagenized super-infective *Salmonella*
typhimurium, "72^{mut}", described above were placed on a rotor
at 37°C, grown to an optical density of 0.4-0.6 at a
30 wavelength of 600nm, and subjected to the chemotaxis
procedures described above.   The chemotaxis cycling procedure
was repeated 4 times through successive challenges with
melanoma-conditioned culture medium.   The population obtained
after 4 cycles was designated #72^{pop-2}.   After the 4th cycling,
35 aliquots of the mixed populations of bacteria were frozen in
glycerol.   Additional aliquots of the mixed population of
Salmonella typhimurium obtained from the fourth cycling were

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then compared to an uncycled mixed population of mutagenized
clone 72 ("72^{mut}") for relative chemotactic abilities toward
control and melanoma-conditioned culture medium. The results
are shown in Table 8.

5

TABLE 8

EVIDENCE FOR POSITIVE CHEMOTACTIC RESPONSES OF *S. TYPHIMURIUM* TO CONDITIONED GROWTH MEDIUM OF CULTURED MELANOMA CELLS[+]

10

Salmonella Strain	Bacteria/Capillary Tube:		
	Control Medium	Conditioned Medium	Ratio:
#72^{mut} (mutagenized, no cycling)	1.2x10^3 ± 0.2	4.4x10^3 ± 2.7	3.7:1
#72^{pop-2} (mutagenized, cycled 4x)	0.5x10^3 ± 0.1	1.8x10^3 ± 0.4	3.6:1

20   [+]Results represent average ± S.D for quadruplicate capillary tubes.

Both populations of bacteria tested showed positive
chemotactic responses to melanoma-conditioned culture medium
over control-conditioned medium, displaying an approximate
4:1 preference for the melanoma-conditioned medium. Although
the chemotactic response of population #72^{pop-2} was not
statistically significant as compared to the chemotactic
response of population #72^{mut} for melanoma conditioned medium,
the chemotactic response of population #72^{pop-2} was
significantly reduced as compared to the chemotactic response
of population #72^{mut} for control medium. Thus, the propensity
of population #72^{pop-2} to enter capillary tubes containing
control medium was significantly reduced. These results
suggest that population #72^{pop-2} is less efficient in motility
generally, however, upon exposure to melanoma-conditioned
medium, population #72^{pop-2} showed a chemotactic response
equivalent to the control population.

Whatever the mechanisms for the different
chemotactic phenotypes expressed by the #72^{mut} and #72^{pop-2}
populations of bacteria in Table 8, the results demonstrate
that the phenotypes can be altered via the selection
5 procedure of exposing bacteria to successive challenges of
melanoma-conditioned media. It is likely that the mixed
populations of mutagenized, chemotactically cycled bacteria
isolated in these experiments contain a number of diverse
mutants expressing likewise diverse phenotypes for the
10 chemotactic response to melanoma cell-conditioned medium.

9.   **EXAMPLE: ISOLATION OF TUMOR-SPECIFIC MUTANTS OF
      SALMONELLA TYPHIMURIUM BY CYCLING *IN VIVO* IN TUMOR-
      BEARING MICE**

Tumor cells inoculated into DBA/2J mice from
15 Cloudman S91 melanoma/macrophage hybrid cell line #48 were
used as the target tumor for the selection of attenuated,
tumor-specific *Salmonella typhimurium*. Super-infective
Salmonella typhimurium clone #72 was mutagenized with
nitrosoguanidine and UVB as described in Section 7.1
20 producing a mutagenized population derived from clone #72.
The mutagenesis procedures produced an increase in the number
of mutations in clone #72 similar to that shown earlier when
the wild type strain #14028 was mutagenized. Cloudman
melanoma/macrophage hybrid #48 cells were inoculated (s.c.)
25 into DBA/2J mice at a concentration of 10^6 cells in 0.1 ml
saline/inoculated site and a total of 4 sites/mouse in the
shoulder and flank regions. After 8-10 days, palpable tumors
developed, and the mice were inoculated (i.p.) with the
mutagenized *Salmonella* population derived from super-
30 infective clone #72. After 2 hours of infection, the mice
were sacrificed, the tumors removed, weighed, and homogenized
in a teflon homogenizer in 5 vol (vol/wt) LB broth. An
aliquot of the homogenate was then diluted about 1:4 in LB
broth, placed on a rotator at 37°C, and incubated through 1-2
35 population doublings, should be monitored at OD^{600}, in order
to ensure the recovery of viable bacteria for successive

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inoculations into tumor-bearing mice. The procedure was
repeated through 4 cycles of infection into mice, followed by
recovery from tumors. At the beginning of each cycle, the
number of bacteria inoculated and the time of infection was
5 reduced from the previous cycle in order to increase the
stringency of selection for tumor-specific mutants. The
resultant population recovered after 4 cycles was designated
#72^{pop-1}. The results of this procedure are detailed in Table
9 below.

10

TABLE 9			
SELECTION FOR MELANOMA-SPECIFIC *SALMONELLA TYPHIMURIUM* IN TUMOR-BEARING MICE			
Infection Cycle	Total # Bacteria Inoculated/mouse	Infection Time	Total # Bacteria Recovered in Tumors*
1	1×10^{10}	120 min	2.1×10^7
2	1×10^9	80 min	1.6×10^6
3	6×10^8	60 min	1.7×10^6
4	2×10^8	40 min	1.4×10^5

*Infecting *Salmonella* were pooled from 4-8 separate tumors for each cycle

These results demonstrate that infecting bacteria can be
25 recovered from tumors *in vivo*. These results also
demonstrate that *in vivo* cycling results in an enriched
population since fewer bacteria were isolated than were
inoculated.

30 10. **EXAMPLE: PROLIFERATION OF *SALMONELLA TYPHIMURIUM* WITHIN MELANOMA CELLS**

Proliferation of a gene-delivering vector within
target tissue can both amplify the gene within the target
tissue as well as allow one to reduce the titer of inoculated
vector, thus reducing the risk of septic shock in the host.
35 The following examples demonstrate that *Salmonella*
typhimurium proliferates in melanoma cells.

10.1.   **PROLIFERATION WITHIN CULTURED HUMAN M2 MELANOMA CELLS**

It was found that *Salmonella typhimurium* proliferated within human M2 melanoma cells in culture with doubling times of about 30 to 60 minutes as illustrated below. Wild type *Salmonella* strain #14028 and super-infective clone #72 were separately introduced into the culture media of human M2 melanoma cells 2 x 10^5 melanoma cells/$25cm^2$ tissue culture flask at 10^6 bacterial c.f.u./ml culture medium. After 1 hour, gentamicin (20 μg/ml) was added to kill external, but not internalized bacteria, and melanoma cells were harvested and assayed for the number of internalized bacteria at the time points indicated. The results are presented in Table 10.

TABLE 10

PROLIFERATION OF *SALMONELLA TYPHIMURIUM* WILD TYPE STRAIN #14028 AND CLONE #72 WITHIN CULTURED HUMAN M2 MELANOMA CELLS[+]

Salmonella Strain	Time (h)	*Salmonella*/10^6 Melanoma Cells	Fold Increase
#14028 wild type	1	6.8 x 10^5	---
	2	1.8 x 10^6	2.6x
	4	1.8 x 10^7	26x
	6	5.4 x 10^7	79x
Clone #72	1	5.8 x 10^6	---
	2	8.0 x 10^6	1.4x
	4	3.2 x 10^7	5.5x
	6	1.4 x 10^8	24x

[+]The numbers represent averages for duplicate and triplicate determinations, with the variation between replicates < ± 25%.

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10.2.   PROLIFERATION WITHIN MELANOMA TUMORS
           GROWN IN MICE

        DBA/2J mice were inoculated s.c. in four areas
(left and right shoulders and flanks) with 10^6 Cloudman S91
melanoma/macrophage hybrid #48 cells. After the appearance
of palpable tumors (8-10 days) the mice were further
inoculated (i.p.) with 2×10^8 *Salmonella typhimurium*. The
Salmonella strains tested were wild type #14028 and super-
infective clone #72. At 4 hours and 21 hours post-
inoculation with bacteria, mice were bled orbitally, and then
euthanized by anesthesia with metofane. Tumors and livers
were removed aseptically, rinsed with sterile NaCl (0.9%),
weighed, and homogenized in LB broth at a ratio of 5:1
(vol:tumor wt). Bacteria were quantitated by plating the
homogenates onto LB plates, incubating overnight at 37°C, and
counting bacterial colonies. Numbers represent averages ±
S.D. The results for the 4 hour and 21 hour incubations of
the bacteria in mice are detailed in Tables 11(A) and 11(B).

TABLE 11

A.   DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 4 HOURS
      FOLLOWING INOCULATION (I.P.) INTO CLOUDMAN S91
      MELANOMA-BEARING DBA/2J MICE

Salmonella Strain	*Salmonella*/ ml Blood	*Salmonella*/ /gm tumor (wet wt)	*Salmonella*/ gm liver (wet wt)	Tumor/ Liver
wild type	6×10^5	$8.9 \pm 2.5 \times 10^4 (n=4)$	3.6×10^5	1:4
clone 72	2×10^5	$3.5 \pm 3.3 \times 10^4 (n=4)$	2.4×10^5	1:7

B.   DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 21 HOURS
      FOLLOWING INOCULATION (I.P.) INTO CLOUDMAN S91
      MELANOMA-BEARING DBA/2J MICE

Salmonella Strain	*Salmonella*/ ml Blood	*Salmonella*/ gm tumor (wet wt)	*Salmonella*/ gm liver (wet wt)	Tumor/ Liver
wild type	1.0×10^4	$1.3 \pm 0.8 \times 10^9 (n=4)$	4.4×10^6	300:1
clone 72	6.7×10^3	$2.1 \pm 2.7 \times 10^9 (n=4)$	5.2×10^5	4000:1

At 4 hours post-inoculation of *Salmonella*, there
were fewer bacteria in the tumors than in the blood stream
and the liver for both wild type clone 14028 and clone 72.
However, by 21 hours, *Salmonella* were found in great
5 abundance in the tumors so that the ratio of bacteria/g
tissue in tumors was 4,000:1 over that in the liver for
super-infective mutant clone 72. After 21 hours post-
inoculation of bacteria, the number of *Salmonella* in the
tumors was similar for both the wild type *Salmonella* strain
10 and clone 72, and was far greater than the total number of
Salmonella originally inoculated, indicating that both wild-
type and clone 72 strains of bacteria proliferated within the
tumors. Thus, the ability of *Salmonella typhimurium* to
infect melanoma cells and proliferate within them was
15 expressed both in cell culture as seen in Table 10 and in
tumors growing in mice as seen in Tables 11A and 11B.

The wild-type strain 14028 showed higher
infectivity in liver than did clone 72. The higher
infectivity of liver by the wild-type *Salmonella* was
20 consistent with the observed greater lethality of the wild
type stain toward DBA/2J mice than that produced by clone 72
at high bacterial inocula ($>10^9$ c.f.u./mouse, data not shown).
Similar results were observed with C57BL\6J mice bearing
B16F10 melanomas as seen in Table 18, Section 15.2.
25 Together, these results demonstrate that selection for
strains of bacteria or other parasites with enhanced tumor
specificity in vitro yields mutant strains with attenuated
host toxicity *in vivo*.

30   10.3.   **DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* IN
                          TUMOR-BEARING MICE**

The following experiments demonstrate that
Salmonella can localize to and proliferate within a tumor of
an animal bearing either multiply-implanted subcutaneous
35 melanoma tumors or naturally occurring metastases.

10.3.1.   DISTRIBUTION OF *SALMONELLA* FOLLOWING
           DIRECT INOCULATION INTO CLOUDMAN S91
           MELANOMA TUMORS

        DBA/2J mice were inoculated s.c. in four areas
(left and right shoulders and flanks) with 10^6 Cloudman S91
melanoma/macrophage hybrid site. Palpable tumors appeared 8-
10 days post-inoculation, representative animals were
selected, and 2 of the 4 tumors (right shoulder and left
flank) were directly inoculated with *Salmonella typhimurium*
super-infective clone #72 at c.f.u.'s of 7×10^4 or 7×10^6
bacteria/tumor. At 21 hours post-inoculation, mice were
euthanized with metofane. Tumors and livers were removed
aseptically, rinsed with sterile NaCl (0.9%), weighed, and
homogenized with NaCl at a ratio of 5:1 (vol:tumor wt).
Bacteria were quantitated by plating the homogenates onto LB
plates, incubating overnight at 37°C, and counting bacterial
colonies. The results are shown in Table 12.

TABLE 12

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* CLONE 72 IN CLOUDMAN S91 MELANOMA-BEARING MICE 21 HOURS FOLLOWING DIRECT INOCULATIONS INTO TUMORS

Inoculum/ tumor	*Salmonella/* g tumor (wet wt)	*Salmonella/* g liver (wet wt)	Tumor/ Liver
7.2×10^4			
Tumor 1*	1×10^9	3.0×10^5	3,300:1
Tumor 2	3×10^7		100:1
Tumor 3	1×10^8		330:1
7.2×10^6			
Tumor 1*	4×10^9	5.0×10^6	800:1
Tumor 2	3×10^9		600:1
Tumor 3	3×10^7		6:1

*inoculated tumor

In summary, two days post-inoculation of super-
infective *Salmonella typhimurium* clone #72 directly into
specificized tumors, the *Salmonella* could be found in distal,
non-inoculated tumors. The amounts of *Salmonella* found in
5   the tumors far exceeded the amounts of *Salmonella* inoculated
into the mice, proving that the *Salmonella* proliferated
within the tumors. The results demonstrate that *Salmonella
typhimurium* can proliferate within a tumor, exit that tumor
via the circulatory system, travel to a distant tumor, and
10 proliferate within that distant tumor.

10.3.2. DISTRIBUTION OF *SALMONELLA* INTO CLOUDMAN S91 MELANOMA METASTASES

This experiment shows that the bacteria should be
15 able to target naturally-occurring metastases of solid
tumors.

1 x 10^5 Cloudman S91 melanoma cells were inoculated
s.c. in the tail of a DBA/2J mouse. After approximately four
weeks, a soft tissue metastasis (~0.5 g) developed with no
20 visible evidence of a primary tumor in the tail.
S. typhimurium clone 72 at 2 x 10^5 c.f.u. was inoculated i.p.
The mouse was sacrificed 48 hours post- inoculation, and the
liver and tumor were removed, homogenized in Luria broth, and
quantitated for *S. typhimurium* by serial dilutions on LB agar
25 plates.

The results shown in Table 12(A) demonstrate that
Salmonella typhimurium clone 72 can target and proliferate
within a metastatic tumor.

30.

35

TABLE 12(A)

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* CLONE 72 IN A DBA/SJ MOUSE WITH A SOFT-TISSUE MELANOMA METASTASIS[*]

Tissue	Salmonella/g tissue	Tumor/Liver
Liver	3.1×10^6	---
Tumor	3.2×10^9	1000:1

[*]Results represent determinations from a single animal.

11. **EXAMPLE: ANTIBIOTIC SENSITIVITY OF WILD TYPE *SALMONELLA TYPHIMURIUM* STRAIN 14028 AND SUPER-INFECTIVE MUTANT CLONE 72**

11.1. **SENSITIVITY TESTED *IN VITRO***

Wild type *Salmonella typhimurium* strain 14028 and super-infective mutant clone 72 were tested for antibiotic susceptibility and were each found to be sensitive to 12 different antibiotics currently used in treating bacterial infections. The bacteria were tested according to the standard protocol to determine antibiotic sensitivity as seen in clinical laboratories, so that a patient is not given an antibiotic to which the microorganism is resistant. The bacteria were tested for antibiotic susceptibility by subjecting them to the Disc Diffusion Susceptibility Technique Kit (Remel Corp., Lenexa, Kansas). The data are presented in Table 13.

TABLE 13

ANTIBIOTIC SENSITIVITY

	Wild Type:	Clone 72:
Ampicillin	S	S
Cefoperazone	S	S
Ceftazidime	S	S
Cefuroxime	S	S
Gentamicin	S	S
Mezlocillin	S	S
Cefazolin	S	S
Ciprofloxacin	S	S
Unasyn	S	S
Ceftriaxone	S	S
TMP/SMX	S	S

11.2.   **SENSITIVITY TESTED *IN VIVO***

Susceptibility of *Salmonella typhimurium* clone 72 to antibiotics was further tested by injecting mice i.p. with bacteria, treating half of the mice with the antibiotic enrofloxacin, and observing the effects of enrofloxacin, an active analog of ciprofloxacin, on the survival of the mice.

Six week old C57B6 female mice were inoculated i.p. with 10^5 cfu *Salmonella typhimurium* clone 72. After four days following inoculation with bacteria, three of the mice were further inoculated i.p. with 100μg/0.1 ml BAYTRIL™ (enrofloxacin), and their drinking water was supplemented with 25μg/ml BAYTRIL™. After 4 days, all the mice were given fresh drinking water without BAYTRIL™. After a total of 21 days, all surviving mice were euthanized and the experiment was terminated. The results are shown below in Table 13(A).

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TABLE 13(A)

SURVIVAL OF C57B6 MICE INJECTED WITH *SALMONELLA TYPHIMURIUM*
CLONE 72 ± BAYTRIL® (enrofloxacin) IN DRINKING H_2O

Conditions	Avg. time of Death ± S.D.
no antibiotic	9.3 ± 5 days
enrofloxacin (days 4-10 post-inoculum)	> 21 days

Mice receiving bacteria only and no antibiotic died after an average of 9 days following inoculation. Mice receiving bacteria followed by antibiotic treatment survived at least 21 days and showed no symptoms of *Salmonella* toxicity when the experiment was terminated. Thus, the results clearly demonstrate that mice can be rescued from *Salmonella*-mediated death by treatment with the antibiotic enrofloxacin. These results are consistent with those presented in Table 13 demonstrating antibiotic sensitivity of *Salmonella typhimurium* strains 14028 and clone 72 by the Disc Diffusion Susceptibility Technique.

The results further underscore the advantage of using antibiotic-sensitive bacteria as vectors in human tumor therapy, since the bacteria can be eliminated by introduction of antibiotics when desired.

12. EXAMPLE: ENHANCED EXPRESSION OF BACTERIAL PROMOTERS IN MELANOMA CELLS

In a preferred embodiment of the present invention an isolated super-infective vector, such as *Salmonella typhimurium* clone 72^{5-3-2} which carries the *HSV* TK gene, the gene is specifically induced in cancerous target cells as opposed to normal cells in the host body. It has been shown that there is a higher relative induction of several

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Salmonella promoter genes, including *pagB* and *pagC*, (Miller
et al., 1989, Proc. Natl. Acad. Sci. USA 86:5054-5058;
Miller et al., 1992, Infect. Immun. 60:3763-3770; Alpuche
Aranda et al., 1992, Proc. Natl. Acad. Sci. USA 89:10079-
5 10083) when the bacteria invade macrophages as opposed to
epithelial cells. In order to test whether these promoters
are also activated when *Salmonella* invade melanoma cells, we
used *Salmonella*-bearing promoter constructs fused to the β-
galactosidase reporter gene.

10   Human melanoma M2 cells, (Cunningham et al, 1992,
Science, 255:325-327) human epithelial 1790 cells and mouse
macrophage cell line J774 cells (American Type Culture
Collection) were seeded at a density of 1 x 10^6 host cells in
25 cm^2 Corning tissue culture flasks. The cells were infected

15 with 5 x 10^7 *Salmonella typhimurium* #14028/ml DMEM culture
medium for 1 hour, washed with fresh medium, and further
cultured for 6 hours with 50 μg/ml gentamicin added to the
culture medium in order to kill the external but not the
internalized *Salmonella*. The melanoma cells were then

20 harvested by scraping them from the substratum in isotonic 1
mM EDTA solution. The cells were pelleted, resuspended in
PBS and an aliquot was removed for quantitation of the
bacteria found within the melanoma cells. The remainder of
the melanoma cells were assayed for β-galactosidase activity.

25   Three *Salmonella typhimurium* clones were used: i)
strain 14028 in which β-galactosidase was constitutively
expressed; ii) strain 14028 in which β-galactosidase was
expressed through activation of the *pagB* promoter; iii)
strain 14028 in which β-galactosidase was expressed through

30 activation of the *pagC* promoter. Thus, through measurements
of β-galactosidase activity, analyses of bacterial *pagB* and
pagC promoter induction in melanoma cells were carried out.
The results are detailed in Table 14.

35

TABLE 14

ENHANCED EXPRESSION OF BACTERIAL PROMOTERS *PAGB* AND *PAGC* IN CULTURED HUMAN MELANOMA M2 CELLS*

Promoter-Induced Activity:Constitute Activity

Cell Line	*pag*B	*pag*C
human epithelial	1.3:1	7.2:1
mouse transformed macrophage	2.9:1	17:1
human melanoma	3.8:1	31:1

*relative activation of *pagB* and *pagC* was assessed through expression of promoter-inducible β-galactosidase activity.

Both the *pagB* and *pagC Salmonella* promoters were induced in human melanoma cells. Levels of induction in melanoma cells were greater than seen in either the epithelial or macrophage cell lines. These data demonstrate that the *pagB* or *pagC* promoter could be used to express genes, such as *HSV* TK or *E. coli* cytosine deaminase, in a melanoma cell-specific manner.

13.   **EXAMPLE: CLONING AND EXPRESSION OF PRODRUG CONVERTING ENZYMES**

The following sections demonstrate useful systems for expression of prodrug-converting enzymes useful for the methods and compositions of the present invention.

13.1.   **CLONING AND EXPRESSION OF *HERPES SIMPLEX* VIRUS THYMIDINE KINASE IN *SALMONELLA TYPHIMURIUM***

Herpes simplex thymidine kinase (*HSV* TK) is known to be an effective pro-drug converting enzyme in the inhibition of melanoma tumor growth (Bonnekoh et al., 1995, J. Invest. Derm. 104:313-317). Accordingly, procedures were carried out to insert an *HSV* TK gene with the β-lactamase signal sequence into both *Salmonella typhimurium* wild type strain 14028 and super-infective tumor-specific mutant clone 72 which is derived from the wild type strain.

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Herpes simplex thymidine kinase cloning by PCR

Plasmid DNA of the vector pHETK2 (Garapin et al.,
1981, Proc. Natl. Acad. Sci. USA 78:815-819) was prepared by
alkaline lysis, phenol/chloroform extraction and ethanol
5 precipitation. PCR primers based on the complete sequence
for the *Herpes simplex* thymidine kinase (McKnight, 1980, Nuc.
A. Res. 8:5949-5964) were: forward 5'-
GATCATGCATGGCTTCGTACCCCGGCC-3' (SEQ ID NO:1) and reverse 5'-
CTAGATGCATCAGTGGCTATGGCAGGGC-3', (SEQ ID NO:2) which
10 corresponds to bases 310-328 (forward) and 1684-1701
(reverse) of the published sequence, with an added sequence
of GATCATGCAT (portion of SEQ ID NO:1) or CTAGATGCAT (portion
of SEQ ID NO:2) (*Nsi*I site and spacer) at the 5'end of each
primer. Each reaction mixture contained 50 ng DNA template,
15 10 pmoles of each primer, 100 mM deoxynucleotide
triphosphates, 1.5 mM Mg^{++} and 0.5 units Taq polymerase
(Perkin Elmer Cetus, Norwalk, CT). Amplification was
performed by 35 cycles of 94°C for 1 minute; 50°C for 15
seconds; 55°C for 1 minute; and 72°C for 2 minutes. The
20 amplified DNA was purified and was cloned into either
pBluescript II KS+ and sequenced with T3 and T7 primers to
confirm the correct DNA had been cloned or was cloned into
p279 cut with *Pst*1 which provides the β-lactamase signal
sequence (Talmadge et al., 1980, Proc. Natl. Acad. Sci. USA
25 77:3369-3373). Transformants were screened using a probe
generated from the original template by random priming
(Boehringer Mannheim, Indianapolis, IN) using [α-^{32}P]dCTP.
Positive clones were further screened by immunoblot.

SDS-PAGE and Immunoblot

30   Sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE) was performed on bacterial lysates
according to Weber and Osbom, 1975, Proteins and sodium
dodecyl sulfate: Molecular mass determination on
polyacrylamide gels and related procedures. In: H. Neurath
35 and R. Hill (eds) The Proteins, Third Edition, vol. 1,
Academic Press, New York pp. 179-223. Immunoblots were
performed according to Towbin et al., 1979, Proc. Natl. Acad.

Sci. USA 76:4350-4354. Primary anti-TK antibodies were
generally used at a 1:1000 dilution. Secondary anti-mouse
antibodies were alkaline phosphatase-conjugates (Promega,
Madison, WI) used at a 1:7,500 dilution, followed by
5 nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl
phosphate (BCIP) colorimetric detection (Promega).

Thymidine Kinase Assay

Bacterial lysates were prepared by pelleting 1 ml
of log-phase bacterial culture for 30 seconds at 12,000 x g
10 in a microfuge centrifuge. The pellet and supernatant were
retained separately and the supernatant was further cleared
by centrifugation for 10 min at 12,000 x g. The pellet was
further treated by resuspension in 100 μl of phosphate
buffered saline containing 1 mg/ml lysozyme and 1% (v/v)
15 Triton X-100 and subjected to three cycles of rapid freezing
and thawing. The resulting material was clarified by
centrifugation at 12,000 x g for 2 minutes. Thymidine kinase
activity was assayed using a modified version of the assay
described by Summers and Summers, 1977, J. Virol. 24:314-318.
20 The reaction mix was incubated at 37°C for 1 hour and then
bound to DE81 paper (Whatman), washed, and the associated
radioactivity determined in a gamma counter.

Salmonella transformation

Transformation of Salmonella strains was performed
25 by electroporation as described by O'Callaghan and Charbit,
1990, Mol. Gen. Genet. 223:156-158. Plasmids transfected
into Salmonella included pHETK2 (Garapin et al., 1981, Proc.
Natl. Acad. Sci. USA 78:815-819) p279 (Talmadge et al., 1980,
Proc. Natl. Acad. Sci. USA 77:3369-3373) and two independent
30 isolates of β-lactamase fusions, p5-3 and p21A-2 (See Figure
4-C for a diagram of p5-3 and p21A-2 where these plasmids are
designated "pTK Sec 1.". Salmonella typhimurium strains
transfected were the wild type 14028 and the super-infective
clone 72.

35   Two independent β-lactamase-TK gene fusion
constructs were isolated and expressed in Salmonella
typhimurium 14028 wild type and clone 72. An immunoblot

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analysis and corresponding enzyme activity assay are
presented in Figures 4A and 4B. All three TK-containing
vectors, the cytoplasmically expressed pHETK2 and the β-
lactamase fusions p5-3 and p21A-2, were detectable by
5 immunoblot and enzyme assay. Relatively little enzyme
activity was recovered from the culture supernatants. Since
the immunoblot analysis shows processing of the signal
sequence, secretion into the periplasmic space of the
Salmonella typhimurium is expected.
10

13.2. SYSTEMS FOR EXPRESSION OF *HERPES SIMPLEX* THYMIDINE KINASE USING VARIOUS PROMOTERS AND SECRETION SIGNALS

A number of constructs were made to express TK
using other promoters and other secretion signals.
15

13.2.1. EXPRESSION AS A *STAPHYLOCOCCUS* PROTEIN A FUSION UNDER THE LACI PROMOTER

Herpes simplex thymidine kinase was amplified by
PCR as described in Section 13.1 above and cloned into the
20 PstI site of pBluescript. This TK clone was subcloned from
bluescript to the *Bam*HI and *Hind*III cite of the secretion
vector pEZZ18 (Promega, Madison, WI; Nilsson and Abrahamsen,
1990, Methods in Enzymology 185:144-161). This resulted in
an in-frame fusion with *Staphylococcus* protein A under the
25 lacI promoter. This plasmid was designated pTK-Sec2 and is
diagramed in Figure 4-C. Plasmid pTK-Sec2 expresses
thymidine kinase as determined by an immunoblot.

13.2.2. CLONING OF THE SERRATIA MARCESENS CHITINASE SIGNAL SEQUENCE AND PROMOTER

30
The promoter and signal sequence of *Serratia
marcesens* chitinase I (Jones et al., 1986, EMBO J. 5:467-473)
was cloned by PCR. The forward and reverse primers had the
following sequence: CTAGACTAGTTTGTCAATAATGACAACACCC (forward)
(SEQ ID NO:3) and GATCGGATCCTTGCCCGGCGCGGCGGCCTG (reverse)
35 (SEQ ID NO:4) which contain *Spe*I and *Bam*HI sites,
respectively. The resulting product was cloned into pSP72

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and confirmed by DNA sequencing. This plasmid was designated pSP-CHT and is also diagramed in Figure 4-C.

13.2.3. EXPRESSION AS A CHITINASE SIGNAL SEQUENCE FUSION UNDER THE CONTROL OF THE CHITINASE PROMOTER

5

Herpes simplex thymidine kinase in pBluescript was subcloned into the pSP-CHT vector using *Bam*HI and *Hind*III. This results in an in-frame fusion with the chitinase signal sequence under the chitinase promoter. This plasmid was

10 designated pTK-Sec3 and is also diagramed in Figure 4-C. Plasmid pTK-Sec3 expresses thymindine kinase as determined by an immunoblot.

13.3. EXPRESSION OF P450 OXIDOREDUCTASE PRODRUG CONVERTING ENZYME IN BACTERIA USING AN EXOGENOUSLY INDUCIBLE PROMOTER

15

The *sulA* promoter element (GENBANK #V00358; Cole 1983) was cloned from *E. coli* genomic DNA by PCR using forward (CTAGAAGCTTATAAGGGTTGATCTTTGTTGTC) (SEQ ID NO:5) and reverse (GTACGATATCCAGAACGATGTGCATAGCCTG) (SEQ ID NO:6)

20 primers which incorporate the *Hind*III and *Eco*RV restriction sites respectively. The PCR conditions were 35 cycles of 95°C, 1 minute; 55°C 1 minute; and 72°C for 1 minute. The product was cloned into pSP72 and sequenced with the T7 primer to confirm that the correct DNA had been obtained.

25 The cloned DNA fragment was 100% identical to the published sequence.

The NADPH-dependent cytochrome p450 oxidoreductase (p450 OR) cDNA clone in the *Eco*RI site of pBluescript that lacks the first initiating ATG (deletion of the first 11

30 nucleotides) of the cDNA described by Yamano et al., 1989, Molecular Pharmacol. 35:83-88, was fused with the *sulA* promoter and initiating sequence by cloning the *sulA* promoter obtained as described above into the *Hind*III and *Eco*RV site of the p450 oxidoreductase gene. The resulting fusion

35 consists of the *sulA* promoter, including the *sulA* ATG (methionine) and subsequent 9 amino acids (YTSGYAHRS) (SEQ ID

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NO:7) as well as six amino acids which follow introduced from
the DNA polylinker and PCR primers (SGYRIP) (SEQ ID NO:8)
followed with the second amino acid of p450 OR, which is G.
This construct, pSP-SAD4-5 is diagramed in Figure 4-D.

5

13.4.   EFFECT OF EXPRESSION OF P450 OXIDOREDUCTASE CONVERSION OF PRODRUG ON BACTERIAL GROWTH

It has been previously shown (Shiba et al., 1959,
Nature 183:1056-1057) that some strains of bacteria are
10 sensitive to low levels of mitomycin. Therefore, to compare
the sensitivity of a specific bacterial strain with and
without the sulA::p450 OR expression plasmid the following
experiment was performed. If the construct is functional,
the presence of the p450 OR gene is expected to result in
15 increased activation of mitomycin resulting in decreased
growth of the bacteria. The sulA::p450 OR expression plasmid
used in this experiment is similar to pSP-SAD4-5 (Section
13.3) except that it is in a pBluescript (pBS) backbone and
has the β-galactosidase transcription unit. This construct
20 is also diagramed in Figure 4-D. The pBS plasmid, with and
without the expression construct, was transfected into
Escherichia coli DH5α by electroporation and clones
containing the correct plasmids were obtained and confirmed
by plasmid isolation and DNA restriction analysis. For each
25 of the two plasmid-bearing strains, a fresh, 4 hr (late log)
culture was diluted 1:100 into LB with 100 μg/ml ampicillin
to select for the presence of the plasmid and grown at 37°C
at 250 rpms. Mitomycin C was added to the cultures in
amounts of 0.0, 0.1 and 0.5 μg per ml.
30   Optical density was determined at 600 nm using a
Perkin Elmer double beam spectrophotometer at 2 and 18 hour
time points. The results are presented in Table 14(A).

35

TABLE 14A

GROWTH OF BACTERIAL CULTURES IN THE PRESENCE OF MITOMYCIN C

OD_{600} t = 2 hours post drug

Amount of Mitomycin C (μg/ml) added

Plasmid	0	0.1	0.5
pBS	0.052	0.050	0.053
sulA::p450 OR	0.037	0.030	0.024

OD_{600} t = 18 hours post drug

Mitomycin C (μg/ml) added

Plasmid	0	0.1	0.5
pBS	2.33	2.23	1.93
sulA::p450 OR	2.26	0.34	0.071

Comparison of the growth of the *E. coli* strain DH5α containing pBS and *sulA*::p450 OR in the absence of drug at the 2 and 18 hour time points shows that the presence of the construct partially inhibits the rate of growth but does not inhibit attaining a high final OD at 18 hours. These data also show that bacteria carrying the pBS backbone plasmid alone are only partially inhibited at the higher mitomycin concentration. However, those carrying the *sulA*::p450 construct show significant inhibition at both early and late time points at both mitomycin concentrations. These data indicate a strong dose response to mitomycin conferred by the presence of the *sulA*::p450 construct.

13.5.   **EXPRESSION OF CYTOSINE DEAMINASE IN**
         SALMONELLA TYPHIMURIUM

E. coli cytosine deaminase (CD) has been shown to be an effective prodrug-converting enzyme useful for gene therapy (Hirschowitz et al., 1995, Human Gene Therapy 6:1055-1063; Huber et al., 1993, Cancer Res. 53:4619-4626; Huber et al., 1994, Proc. Natl. Acad. Sci. USA 91:8302-8306; Moolten,

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1994, Cancer Gene Ther. 1:279-287; Mullen et al., 1992, Proc. Natl. Acad. Sci. USA 89:33-37; Mullen et al., 1994, Cancer Res. 54:1503-1506; Trihn et al., 1995, Cancer Res. 55:4808-4812). CD functions by converting the non-toxic 5-

5 fluorocytosine (5-FC) to the toxic compound 5-fluorouracil (5-FU). *Salmonella* possess an endogenous CD, however, its expression is catabolite repressed (West and O'Donovan, 1982, J. Bacteriol. 149:1171-1174). A CD expression vector using the constitutively active β-lactamase promoter to ensure

10 expression of CD within tumors was cloned as described below.

Cloning and expression of CD

PCR primers based on the complete sequence for *E. coli* cytosine deaminase (Huber et al., 1993, Cancer Res. 53:4619-4629) were forward: 5'-GATCATGCATGTGGAGGCTAACAGT-3' (SEQ ID NO:9) and reverse: 5'-CTAGATGCATCAGACAGCCGCTGCGAAGGC-

15 3' (SEQ ID NO:10), corresponding to the published sequence, with the added sequence GATCATGCAT (portion of SEQ ID NO:9) or CTAGATGCAT (portion of SEQ ID NO:10) which is a *Nsi*I site and spacer at the 5' end of each primer. Each 25 μl reaction

20 mixture contained 50 ng DNA template, 10 pmoles of each primer, 100 mM deoxynucleotide triphosphates, 1.5 mM Mg and 0.5 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Amplification was performed by 35 cycles of 94°C for 1 minute; 50°C for 15 seconds; 55°C for 1 minute; and 72°C for

25 2 minutes. The amplified DNA was purified on an agarose gel and the band of correct size was cloned into 1) pBluescript II KS+ and sequenced with T3 and T7 primers to confirm the correct DNA had been cloned and 2) p279 cut with PstI which provides the β-lactamase signal sequence and the constitutive

30 β-lactamase promoter. This second construct was designated pCD-Sec1 and is diagramed in Figure 4-E. Transformants were screened using a [α-^{32}P]dCTP-labeled oligonucleotide probe. Positive clones were further screened by immunoblot using anti-CD antibodies described below.

35   Primary Antibodies to Cytosine Deaminase

CD was subcloned from pBluescript into pGEX I and expressed using IPTG. The expressed protein was found to be

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insoluble and present in inclusion bodies. CD-glutathione-S-
transferase (GST) fusion protein was purified from inclusion
bodies by washing in 0.1% w/v Triton X-100, repelleted and
resuspended in SDS-PAGE sample buffer. The material was
5 separated on a 3 mm preparative 10% polyacrylamide gel and
excised after visualization with 3M potassium acetate at 4°C.
The purifed bands were homogenized and injected i.p. into
DBA2J mice with Freund's complete (day 0) and incomplete (day
14) adjuvant. After 6 weeks the mice were bled and the
10 ability of the serum antibodies to bind to cloned CD
confirmed by Immunoblot.

SDS-PAGE and Immunoblot

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
was performed on bacterial lysates according to Weber and
15 Osbom, 1975, Proteins and sodium dodecyl sulfate: Molecular
mass determination on polyacrylamide gels and related
procedures. In:H. Neurath and R. Hill (eds) The Proteins,
Third Ed., Vol. I, Academic Press, New York, pp. 179-223.
Immunoblots were performed according to Towbin et al., 1979,
20 Proc. Natl. Acad. Sci. USA 76:4350-4354. Primary anti-CD
antibodies described above were generally used at a 1:500
dilution. Secondary anti-mouse antibodies were alkaline
phosphatase-conjugates (Promega, Madison, WI) used at a
1:7,500 dilution, followed by nitroblue tetrazolium (NBT) and
25 5-bromo-4-chloro-indolyl phosphate (BCIP) colorimetric
detection (Promega, Madison, WI).

CD Enzyme Assay

Bacterial lysates were prepared by pelleting 50 ml
of overnight bacterial culture at 3000x g for 10 minutes and
30 resuspending them in 2.5 ml of PBS. The cells were sonicated
and the debris removed by pelleting in a microfuge at 12,000x
g for 10 minutes. The enzyme assay performed was modified
from Mullen et al., 1992, Proc. Natl. Acad. Sci. USA:89:33-
37. 10 μl of cell extract was incubated with 1 μl [H^3]-5FC (1
35 μCI/μl) a 37°C. 1 μl was spotted on a Kodak 13254
microcrystalline nitrocellulose TLC plate, Eastman Kodak,
Rochester New York, and separated using 95:5 Butanol:water

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with unlabeled 5FC and 5FU markers.   The plates were cut
based upon separation of the marker lanes and quantified
using a liquid scintillation counter.

                    Salmonella transformation

5   Transformation of *Salmonella* strains was performed
by electroporation as described by O'Callaghan and Charbit,
1990, Mol. Gen. Genet. 223:156-158.   Plasmids transfected
into *Salmonella* were p279 and pCD-Sec1.   *Salmonella
typhimurium* strains transfected were strains YS721, YS7211,
10 YS7212 and YS7213 which are described *infra* in Section 18.
E. coli strains transfected were strains DH5α and KL498
(Δ*cod*).

          Biodistribution of Salmonella carrying the CD expression
construct

15   *Salmonella typhimurium* clone YS7212 carrying the CD
expression construct pCD-Sec1 was grown in LB media to an
OD_{600} of 0.8.   An aliquot of 1.0×10^6 bacteria were inoculated
i.p. into C57/B6 mice which had been implanted with 2×10^5
B16 melanoma cells 16 days prior to the bacterial infection.
20 At two days post bacterial infection, mice were sacrificed
and tumors and livers assayed for the presence of the
bacteria by homogenization and plating of serial dilutions.

               The expressed protein product of pCD-Sec1 bound to
the anti-CD anti-serum by immunoblot analysis.   When this
25 clone was transferred to the *E. coli* strain KL498 which lacks
CD, it was found to confer a high degree of enzyme expression
as determined by the conversion of 5FC to 5FU, as shown in
Figure 4-F.   Figure 4-F also demonstrates that the cloned CD-
expression plasmid gives higher levels of conversion than the
30 *E. coli* strain MG1655 which expresses the wild type haploid
cod gene which encodes for endogenous CD.

     14. **EXAMPLE: PROLIFERATION OF *SALMONELLA TYPHIMURIUM*
          CLONE #72[5-3-2] IN MELANOMA TUMORS IN MICE**

35   In a similar set of experiments as in Section 10.2,
DBA/2J mice (approximately 10 weeks) were inoculated (s.c.)
with 3×10^5 Cloudman S91 melanoma/macrophage hybrid #48 cells

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in each of 4 sites over the right and left shoulders and
flanks. Tumors were palpable 10-12 days post inoculation of
tumor cells from tissue culture into mice. After two weeks
post-inoculation of tumor cells (s.c.), tumor-bearing mice
5   were additionally inoculated (i.p.) with 2×10^5 c.f.u. of *S.
typhimurium* clone 72 containing the *HSV TK* gene with the β-
lactamase signal sequence which is designated 72^{5-3-2}. After 2
and 10 days of bacterial infection without antibiotic
treatment, representative tumor-bearing animals were
10 sacrificed and their tumors and livers were homogenized and
quantitated for c.f.u. of *Salmonella* per gram of tissue. In
addition, individual clones of bacteria were isolated from
the liver and tumor homogenates 10 days post-infection and
tested for the genetic markers *xyl*^{neg} (inability to metabolize
15 xylose, characteristic of clone #72) and *tet*^{res} (resistance to
the antibiotic tetracycline). The genotype of the inoculated
Salmonella typhimurium clone #72^{5-3-2} was *xyl*^{neg} and *tet*^{res}. The
tet^{res} clones of *Salmonella* were assumed to carry the HSV TK
gene, since the HSV TK gene was carried on a plasmid that
20 carried the *tet*^{res} marker.

The results are presented in Tables 15 and 16.
After 2 days of infection, the tumors contained an average of
1.5×10^9 *Salmonella*/g tumor and 2.0×10^5 *Salmonella*/g liver,
with an average ratio of tumor:liver of about 7,500:1.

25

30

35

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TABLE 15

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 2 DAYS FOLLOWING
INOCULATION (I.P.) INTO CLOUDMAN S91 MELANOMA-BEARING DBA/2J
MICE

Tissue	*Salmonella*/g Tissue	Tumor/Liver
Liver (n = 2)	2.0×10^5	---
Tumor (n = 4)	$1.5 \pm 0.9 \times 10^9$	7,500:1

After 10 days of infection (Table 16), the tumors
contained an average of 2.9×10^9 *Salmonella*/g tumor and 2.7×10^5 *Salmonella*/g liver, a ratio of 11,000:1 (tumor:liver),
similar to the distribution of bacteria seen 1-2 days post-
infection. These results demonstrate that once inoculated
(i.p.) into tumor-bearing mice, *Salmonella* enter the
circulatory system, infect the tumor cells, proliferate
within the tumors, and exist there in a compartmentalized
fashion.

TABLE 16

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 10 DAYS FOLLOWING
INOCULATION (I.P.) INTO CLOUDMAN S91 MELANOMA-BEARING DBA/2J
MICE

Tissue	*Salmonella*/gm Tissue	Tumor/Liver	xylose	tetracycline
Liver	2.7×10^5	---	neg	9/10 res
Tumor #1	4.2×10^9	16,000:1	neg	3/7 res
#2	3.1×10^9	12,000:1	neg	7/8 res
#3	1.3×10^9	4,800:1	neg	8/8 res
Average	2.9×10^9	11,000:1		

The results further demonstrate that 10 days post-
infection, all of the bacterial clones examined were *xyl*[neg],
proving their genetic relationship to the inoculated clone

#72[5-3-2], and that 27/33 clones remained *tet*[res], demonstrating
high degree of retention (82%) of the *HSV* TK containing
plasmid within the host bacteria. In experiments not shown
here, the same plasmid was found to be 100% retained after 42
5 hours of infection in tumor-bearing mice.

In a continuation of the above experiments
summarized in Tables 14, 15 and 16, the *Salmonella* infections
in melanoma-bearing mice were continued for a total of 4
weeks. To alleviate the symptoms of *Salmonella* poisoning
10 (shaking, matted hair) the animals were placed on antibiotics
for the final two weeks. Such antibiotic treatments
consisted of the inclusion in the mouse drinking water of
SULFATRIM™ Pediatric Suspension (Schein Pharmaceutical, Inc.;
sulfamethoxazole 40 mg/ml, trimethoprim 8 mg/ml) at a final
15 concentration of 15 ml SULFATRIM™/500 ml drinking water. At
termination of the experiment, the surviving mice were
sacrificed by euthanasia, and the tumors and livers were
removed. Portions of the tissues (1-2 mm^3) were fixed in
formalin and stained for histological examination. The
20 remaining portions were weighed, homogenized in 5 ml LB
broth/g tissue, and the number of *Salmonella* were quantitated
on LB agar plates. Results are presented in Table 17.

TABLE 17

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 4 WEEKS FOLLOWING INOCULATION (I.P.) INTO MELANOMA-BEARING DBA/2J MICE

Tissue	*Salmonella*/g tissue	Tumor/Liver
Liver	5.1×10^7	---
Tumor		
#1	2.2×10^9	43:1
#2	9.4×10^8	18:1
#3	2.3×10^9	45:1
#4	2.0×10^8	6:1
Average	$1.4 \pm 1 \times 10^9$	28:1

The excised melanoma tumors averaged less than 1 gram in weight compared to 5-10 gm tumors in control animals at death (data not shown). It was found that these tumors contained an average of 1.4 x 10^9 Salmonella/g tumor, similar

5 to the number of tumor-infecting bacteria seen at 1, 2, and 10 days post-inoculation. However, the number of Salmonella in the liver increased during the 4 week infection, so that the average ratio of bacteria in tumor over liver was reduced to 28:1 compared to the ratios obtained with infection

10 periods of up to 10 days as seen in Tables 14, 15 and 16.

15. **EXAMPLE: MICROSCOPIC DETECTION OF SALMONELLA TYPHIMURIUM IN MELANOMAS IN VIVO**

15.1. **DETECTION OF SALMONELLA TYPHIMURIUM**
15 **WITHIN CLOUDMAN S91 MELANOMAS GROWING IN DBA/2J MICE**

In order to study the histopathology of Salmonella infection in the tumor-bearing mice, representative melanoma tumors were removed from euthanized mice with or without

20 Salmonella infection. Portions of the tissues (1-2 mm^3) were fixed in formalin, embedded and sectioned, and the sections stained with either hematoxylin and eosin, or tissue gram stain for histological examination. Results of these studies are shown in Figures 5A-B.

25 Figures 5A-B are photomicrographs of histological sections from a Cloudman S91 melanoma/macrophage hybrid #48 melanoma growing subcutaneously in a DBA/2J mouse. The tumor was excised from a mouse that had been inoculated 2 days earlier with 3 x 10^5 c.f.u. Salmonella typhimurium super-

30 infective clone 72 carrying the HSV TK gene, 72^{5-3-2}. A portion of the tumor was weighed, immersed in LB at 5ml/g tumor, homogenized with a ground glass homogenizer, and the tumor homogenate was plated onto LB-Agar culture plates at various dilutions in order to quantitate the amount of

35 Salmonella typhimurium in the tumor. Quantitation of the bacteria revealed that the tumor contained 1.4 x 10^9 Salmonella/g. Figure 5A. A section stained with hematoxyn

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and eosin shows a cross-section of the tumor with an area of
necrosis, denoted by the arrow. Figure 5B. A section
stained with tissue gram stain shows gram negative bacteria
in an area of necrosis area of the tumor. When viewed with
5 the light microscope, the bacteria stain pink/purple against
a yellow background. *Salmonella*-infected necrotic areas were
surrounded with dead tumor cells that did not stain with
tissue gram stain but which could be detected through
melanin-containing melanosomes (see Figure 6). These results
10 show that the necrotic areas of solid tumors are accessible
to *Salmonella* when the bacteria are introduced into a tumor-
bearing host via the circulatory system.

In an additional set of analyses, sections of
Cloudman S91 melanoma/macrophage hybrid #48 melanoma tumors
15 growing in a *Salmonella*-infected mouse were examined with the
electron microscope. To initiate the experiment, a mouse was
inoculated s.c. with 8 x 10^5 tumor cells. A palpable tumor
mass was detected 11 days later, at which time the mouse was
inoculated i.p. with 3.6 x 10^6 c.f.u. of *S. typhimurium* super-
20 infective clone #72. Forty-two hours post-inoculation, the
mouse was sacrificed by metofane anesthesia. The tumor was
excised using aseptic techniques. Quantitation of the
bacteria within the tumor revealed that the tumor contained
approximately 7.5 x 10^9 *S. typhimurium*/g upon excision at 42
25 hours. In contrast, the concentration of *S. typhimurium* in
the liver from the same mouse was approximately 2.0 x 10^7/g, a
ratio of bacteria in tumor to liver of approximately 400:1.

A second portion of the tumor was cut into 1-2 mm^3
pieces and fixed in 1/2 strength Karnovsky's fixative for 6
30 hours at 4°C, followed by washing in cacodylate buffer
overnight. The tumor tissue was post-fixed with 1% OsO_4 and
1.5% potassium ferrocyanide in cacodylate buffer for 2 hours
and embedded in Spurr's resin. Ultrathin sections were
stained with uranyl acetate and lead citrate. They were
35 viewed with a Zeiss 109 electron microscope.

Shown in Figure 6 is an electron micrograph of a
field within a melanoma tumor that includes two separate *S.*

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typhimurium along with numerous melanosomes, which are specialized subcellular organelles present in the cytoplasm of melanoma cells. The presence of bacteria along with the melanosomes provides proof that the *S. typhimurium* entered

5 the cytoplasm of the melanoma cell via the bloodstream of the mouse. The *S. typhimurium* in the electron micrograph appear identical to those shown previously in intestinal epithelial cells following an experimental infection of the mouse, Takeuchi, 1967, Am. J. Pathol. 50:109-1361.

10   In summary, i) examination with the light microscope revealed that *Salmonella typhimurium* exists in the necrotic areas of Cloudman S91 melanomas growing in infected DBA/2J mice; and ii) examination with the electron microscope revealed that *Salmonella typhimurium* also exists within the

15 cytoplasm of melanoma tumor cells.

15.2.   DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* WITHIN MOUSE B16F10 MELANOMA TUMORS GROWN IN C57BL/6J MICE

C57BL/6J mice (11-13 weeks old) were inoculated

20 s.c. in two sites (shoulder and flank), with 3.5 x 10^5 B16F10 mouse melanoma cells per site. After the appearance of palpable tumors (approximately 2 weeks) the animals were further inoculated i.p. with about 10^5 bacteria of the following three strains: i) wild type *Escherichia coli* K-12

25 strain #CSH 101; ii) *Salmonella typhimurium* strain 14028; and iii) mutant *Salmonella typhimurium* super-infective clone 72 carrying the *HSV* thymidine kinase gene, 72^{5-3-2}. After about 2 days of infection, mice were euthanized by anesthesia with metofane. Tumors and livers were removed aseptically, rinsed

30 with sterile 0.9% NaCl, weighed, and homogenized in LB broth at a ration of 5:1 (vol. broth:wt. tumor). Prior to homogenization, 1-2 mm^3 pieces of tissue were removed from representative tumors, fixed with 1/2 strength Karnovsky's fixative, and processed for analysis with the electron

35 microscope. Bacteria in the homogenates were quantitated by

plating onto LB plates, incubating overnight at 37°C, and counting bacterial colonies.

Results were as follows: i) wild type *E. coli* were found in relatively low numbers in both the tumor and liver
5 of the inoculated animals at concentrations averaging <10^3/g tumor and <10^2/g liver. ii) wild type *S. typhimurium* were found in significantly higher numbers than *E. coli* in both tumor and liver, with infecting bacteria ranging from 2 x 10^7 to 6 x 10^8 c.f.u./g tumor, and 4 x 10^6 c.f.u./g liver. One of
10 the two C57BL/6J mice inoculated with the wild type *S. typhimurium* strain died, possibly from septic shock. iii) *S. typhimurium* super-infective clone 72 were also found in significantly higher numbers than *E. coli* in both tumor and liver, further, the number of clone 72 *S. typhimurium*/g liver
15 was significantly lower than the number of wild type *S. typhimurium*/g liver. The results are detailed below in Table 18.

TABLE 18

20 **DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI* 2 DAYS FOLLOWING INOCULATION (I.P.) INTO C57B6 MICE BEARING B16F10 TUMORS**

Bacterial Strain	Mouse	Tissue	Bacteria/ gm Tissue	Tumor/ Liver
E. coli K-12 (CSH #101)	A	Liver	355	—
		Tumor #1	1200	4:1
		Tumor #2	50	1:7
	A'	Liver	100	—
		Tumor #1	50	1:7
S. typhimurium (14028 wild type)	B	Liver	4.3 x 10^6	—
		Tumor #1	2.3 x 10^7	5:1
		Tumor #2	6.0 x 10^8	136:1
	B'	(dead)	—	—

S. typhimurium (clone # 72^{5-3-2})	C	Liver	2.0×10^4	—
		Tumor #1	1.0×10^8	5,000:1
		Tumor #2	1.2×10^5	6:1
	C'	Liver	8.5×10^4	—
		Tumor #1 (2.5g)	9.3×10^8	11,000:1

In summary, *Salmonella typhimurium* displays natural capabilities over *Escherichia coli* in its ability to infect and proliferate within B16F10 melanoma tumors. Furthermore, super-infective clone 72^{5-3-2} displays superior qualities to its wild type parental strain 14028 in its reduced infection of liver in C57BL/6J mice, *i.e.*, the wild-type strain 14028 showed greater infectivity toward liver than did clone 72^{5-3-2}. The higher infectivity of liver by the wild-type *Salmonella* was consistent with the observed greater lethality of the wild type stain toward DBA/2J mice and the greater infectivity of liver in DBA/2J mice than that produced by clone 72 as seen in Table 11B. Together, the results in Tables 11B and 18 provide the first evidence that selection for strains of bacteria or other parasites with enhanced tumor specificity *in vitro* can yield mutant strains with attenuated host toxicity *in vivo*.

15.3.   **MICROSCOPIC DETECTION OF *SALMONELLA TYPHIMURIUM* WITHIN B16F10 MELANOMAS GROWING IN C57BL/6J MICE**

Representative B16F10 melanoma tumors were removed from euthanized mice with or without *Salmonella* infection. Portions of the tissues (1-2 mm³) were fixed in formalin, embedded and sectioned, and the sections stained with either hematoxylin and eosin, or tissue gram stain for histological examination. Results of these studies are shown in Figures 7A-B. Figures 7A and 7B are light micrographs of histological sections from a B16F10 melanoma growing

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subcutaneously in a C57BL/6J mouse. The tumor was excised
from a mouse that had been inoculated 2 days earlier with 2 x
10^5 c.f.u. *Salmonella typhimurium* super-infective clone, 72^{5-3-2}
carrying the *HSV* TK gene. Quantitation of the bacteria

5 within the tumor revealed that the tumor contained
approximately 9 x 10^8 c.f.u. *S. typhimurium*/g upon excision 2
days post-infection with bacteria. In contrast, the
concentration of *S. typhimurium* in the liver from the same
mouse was approximately 2.0 x 10^5/g, a ratio of bacteria in

10 tumor to liver of approximately 400:1. Figure 7: A section
stained with tissue gram stain shows gram negative bacteria
in an area of necrosis within the tumor. The infected
necrotic area is surrounded by dead melanoma cells that do
not stain with the tissue gram stain but which appear brown

15 in color due to the presence of melanized melanosomes. When
viewed with the light microscope, the bacteria stain
pink/purple against a yellow background. The results show
that necrotic areas of B16 melanoma tumors are accessible to
Salmonella when the bacteria are introduced into a tumor--

20 bearing host via the circulatory system.

         A second portion of the above-described tumor was
cut into 1 mm³ pieces and fixed in 1/2 strength Karnovsky's
fixative for 6 hours at 4°C, followed by washing in
cacodylate buffer overnight. The tumor tissue was post-fixed

25 with 1% OsO_4 and 1.5% potassium ferrocyanide in cacodylate
buffer for 2 hours, and embedded in Spurr's resin. Ultrathin
sections were stained with uranyl acetate and lead citrate.
They were viewed with a Zeiss 109 electron microscope as
depicted in Figure 8.

30   The electron micrograph in Figure 8 shows numerous
Salmonella typhimurium in extracellular spaces, denoted by
arrows, in an area of necrosis. A single bacterium is also
seen within the cytoplasm of a dying melanoma cell. The
cytoplasm of the dying melanoma cell also contains numerous

35 black melanosomes, characteristic of the B16F10 melanoma.
         The *S. typhimurium* in the electron micrograph
appear identical to those shown previously in intestinal

epithelial cells following an experimental infection of the
mouse, Takeuchi, 1967, Am. J. Pathol. 50:109-136.

In summary, i) examination with the light
microscope revealed that *Salmonella typhimurium* exist\
5 abundantly in the necrotic areas of B16F10 melanomas growing
in infected B16F10 mice; and ii) examination with the
electron microscope revealed that *Salmonella typhimurium* also
exist within the cytoplasm of tumor cells. *Salmonella* were
also observed in tumor-associated neutrophils.
10

16.   **EXAMPLE: USE OF SUPER-INFECTIVE TUMOR-SPECIFIC
GENE-DELIVERING *SALMONELLA TYPHIMURIUM* FOR
TREATMENT OF MICE BEARING MELANOMA TUMORS**

16.1.   **TREATMENT OF CLOUDMAN 591 MELANOMA**
15   *Salmonella typhimurium* super-infective mutant 72^{5-3-}
^{2}, constitutively expressing the *Herpes simplex* virus
thymidine kinase gene with the β-lactamase signal sequence,
was used for gene therapy of melanoma in mice (see Figure 4-
C). DBA/2J mice (approximately 10 weeks) were inoculated
20 (s.c.) with 3×10^5 Cloudman S91 melanoma/macrophage hybrid
cells in each of 4 sites over the right and left shoulders
and flanks. Tumors were palpable 10-12 days post-inoculation
of tumor cells.

After two weeks post-inoculation of tumor cells,
25 tumor-bearing mice were further inoculated (i.p.) with 2×10^5
c.f.u. of *S. typhimurium* clone 72 containing the *HSV*
thymidine kinase gene with the β-lactamase secretory signal
sequence which is designated 72^{5-3-2}. Twelve hours after
inoculation of the bacteria, some of the mice were further
30 inoculated (i.p.) with 2.5 mg ganciclovir sodium (Cytovene,
Syntex Laboratories, Palo Alto, Calf.) in isotonic saline.
These same mice received this dosage of ganciclovir four
times over a 3 day period. Control tumor-bearing mice also
received ganciclovir but no bacteria. Another set of tumor-
35 bearing mice was inoculated with bacteria, but received no
ganciclovir. At various times appropriate groups of mice,
treated as above, were also given the antibiotic Sulfatrim™

Pediatric Suspension (Schein Pharmaceutical, Inc.;
sulfamethoxazole 40 mg/ml, trimethoprim 8 mg/ml) at a
concentration of 15 ml Sulfatrim™/500 ml drinking water.

Results were as follows:

5   1)   Control melanoma tumor-bearing mice, receiving
ganciclovir and antibiotic treatment (Sulfatrim™ in drinking
water) but no bacteria, developed rapidly growing tumors that
initially doubled in size every 3 to 4 days, determined by
caliper measurements as shown in Figure 9. These animals

10 exhibited little or no side-effects from the ganciclovir
treatment, confirming previous reports on the minimal
toxicity of the ganciclovir pro-drug in mice in the absence
of a suitable thymidine kinase converting enzyme (Bonnekoh et
al, 1995, J. Invest. Dermatol. 104:313-317). By 30 days

15 post-inoculation with tumor cells, all mice in this group had
formed massive subcutaneous tumors (5-10 gm) and had died
from melanoma.

2)   One group of tumor-bearing mice received
bacteria for a total of 10 days without administration of

20 antibiotics, and received no ganciclovir. These animals had
tumors that were significantly reduced in size from the
tumors in control mice (Figure 10). The effect of Salmonella
alone on reducing tumor size became evident several days
after the effect of Salmonella plus ganciclovir on tumors had

25 been observed as described below. However, all the animals
in the "Salmonella alone" group developed symptoms of
Salmonella infection (shaking, matted hair) and 50% of these
animals succumbed between 5-10 days post-infection. The
remaining animals were treated with the antibiotic Sulfatrim™

30 (Schein Pharmaceutical, Oral Suspension) at a concentration
of 15 ml/500 ml drinking water. This treatment reduced the
clinical symptoms of Salmonella infection in the mouse
population within 24-48 hours. The surviving animals from
this protocol had significantly smaller tumors than control

35 animals and remained alive past the 30 day period, when all
of the control animals had died from melanoma.

3)   Another group of tumor-bearing mice received ganciclovir plus bacteria during a 4-day treatment period. About 50% of the animals succumbed within 1-2 days of this treatment, apparently from the conversion of ganciclovir to

5   its toxic, phosphorylated form by the *HSV* TK expressed by the *Salmonella* clone 72^{5-3-2} within the body of the mouse. At this time ganciclovir treatment was discontinued and the surviving animals were placed on Sulfatrim™ antibiotic to control the *Salmonella* infection. The total time of exposure to

10 *Salmonella* without antibiotic was 4 days. The survivors from this protocol had significantly smaller tumors than control animals and remained alive past the 30 day period when all the control animals had died from melanoma (Figure 11).

In a further set of experiments, tumor progression

15 was measured with calipers in various treated and untreated tumor-bearing mice. Groups of mice bearing Cloudman S91 melanoma/macrophage hybrid #48 melanoma tumors as described above were inoculated (i.p.) with 3 x 10^5c.f.u. *Salmonella typhimurium* super-infective clone 72 carrying the *Herpes*

20 *simplex* virus thymidine kinase gene, 72^{5-3-2}. Twenty-four hours after inoculation with bacteria, the mice were further inoculated with ganciclovir at doses of 2.0 mg, with a total of 6 inoculations over a 5 day period. The mice were then subjected to antibiotic treatment with a combination of 15

25 ml/500 ml Sulfatrim™ and 20 µg/ml Baytril™ (Miles) in their drinking water. Baytril™ or enrofloxacin is 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid. Tumor growth was assessed with periodic caliper measurements of tumor length, width, and

30 height, and computed as tumor volume in mm^3 by techniques known to the science of tumor biology. Results were plotted on a semi-logarithmic scale and generation times, the time in hours for one doubling in volume, were calculated. The following formula was used:

35   Generation time = 0.69 (t)/In (T_1/T_2), where t equals the time in hours between the initial tumor volume (T_1)

and the final tumor volume (T_2) over the linear portion of the curve.

The results are shown in Figure 9 and Table 19. Mean doubling times of tumors in untreated control mice and 5 mice treated with ganciclovir but no *Salmonella* infection were similar, being 83 and 94 hours respectively. Tumors in mice treated with *Salmonella* for 5 days but no ganciclovir doubled at a mean rate of 125 hours. Tumors in mice treated with *Salmonella* for 5 days as well as ganciclovir showed no 10 growth over the 10 day measurement period, and in some cases regressed with the treatment.

TABLE 19

EFFECTS OF *HSV TK*-CONTAINING *SALMONELLA TYPHIMURIUM* ON THE GROWTH OF CLOUDMAN S91 MELANOMAS IN DBA/2J MICE ± TREATMENT WITH GANCICLOVIR

Treatment	Mean Tumor Doubling Time (hrs)
none	83
ganciclovir	94
S. typhimurium	125
S. typhimurium + ganciclovir	no growth

In summary: a) Control tumor-bearing animals receiving ganciclovir and antibiotic treatment, but no *Salmonella*, succumbed from massive tumors within 30 days of inoculation of tumor cells; b) Animals receiving *Salmonella* alone followed by antibiotic treatment showed reduced tumor 30 growth rate and prolonged survival over control animals; c) Animals receiving a combination of ganciclovir and *Salmonella* followed by antibiotic treatment showed little or no tumor growth compared to control animals, and prolonged survival over control animals. The results indicate that 35 *Salmonella typhimurium* expressing the *Herpes simplex* virus thymidine kinase gene was able to convert ganciclovir to its

phosphorylated form within the melanoma tumors, thus reducing
tumor size and prolonging survival of the mice.

16.2. TREATMENT OF B16F10 MELANOMA

5   C57B6 mice were inoculated s.c., left shoulder
region, with 5×10^5 B16F10 melanoma cells from culture. At 8
days post-tumor implantation some of the mice were further
inoculated i.p. with 2×10^6 c.f.u. attenuated *Salmonella
typhimurium* strains YS721, YS7211, YS7212 or YS7213 (see

10 Section 18, *infra*) each carrying the HSV TK gene. At 11 days
post-tumor implantation, GCV (ganciclovir sodium, CYTOVENE™,
Syntex Laboratories, Palo Alto, CA.) was inoculated i.p. into
groups of mice (n=5 or n=10) under the following treatment
protocols: a) total dose = 7.5 mg/mouse (2.5 mg day 11, 1.25

15 mg day 12; 2.5 mg day 18, 1.25 mg day 19); b) total dose =
5.0 mg/mouse (2.5 mg day 11, 2.5 mg day 12); c) total dose =
3.75 mg/mouse (2.5 mg day 11, 1.25 mg day 12); d) total dose
= 2.5 mg/mouse (1.25 mg day 11, 1.25 mg day 12); e) total
dose = 1.25 mg/mouse (1.25 mg day 11). At 18 days post-tumor

20 implantation (10 days post bacterial inoculation) all animals
were given enrofloxacin antibiotic, 0.2 mg/ml, in their
drinking water (BAYTRIL™) and maintained with this antibiotic
supplement for 2 weeks. Tumor growth was assessed by caliper
measurements and computed as volume in mm^3. Animals were

25 euthanized and listed as dead when the sum of their tumor
measurements, length + width + height, reached 60 mm, or when
they became moribund (listless, cessation of drinking).
           The results obtained are illustrated in Figure
11C-H in Table 19(A).

30

35

TABLE 19(A)

SURVIVAL OF C57B6 MICE INOCULATED WITH *SALMONELLA TYPHIMURIUM* ± HSV TK GENE: EFFECTS OF GANCICLOVIR

Strain	(n=)	GCV	Time Days ± S.D.	Treated/Control T/C
Control	(10)	-0-	25 ± 0	1.0
	(10)	3.75mg	27 ± 1	1.1
YS7212	(10)	-0-	42 ± 2	1.7
YS7212	(10)	3.75mg	33 ± 4	1.3
YS7212/p5-3	(10)	-0-	45 ± 3	1.8
YS7212/p5-3	(10)	3.75mg	40 ± 4	1.6
YS7213	(10)	-0-	34 ± 2	1.3
YS7213	(10)	3.75mg	35 ± 2	1.4
YS7213/p5-3	(10)	-0-	29 ± 2	1.2
YS7213/p5-3	(10)	3.75mg	33 ± 2	1.3
YS7211	(10)	-0-	40 ± 4	1.6
YS7211	(10)	3.75mg	35 ± 4	1.4
YS7211/p5-3	(10)	-0-	34 ± 2	1.4
YS7211/p5-3	(5)	1.25mg	30 ± 4	1.2
	(5)	2.50mg	37 ± 4	1.5
	(5)	3.75mg	38 ± 4	1.5
	(5)	5.0mg	42 ± 6	1.7
	(5)	7.5mg	39 ± 6	1.6

*Time of death post tumor cell inoculation.

The results from the various treatment protocols for the B16F10 melanoma-bearing mice were as follows:

1)   Effects of GCV on tumor-bearing animals
       with no bacterial inoculation

Mice receiving melanoma cells but no bacteria were treated with GCV on days 11-12 post inoculation with tumor cells at doses from 3.75 mg-10 mg/mouse, depending on the experiment. In all trials, mice treated with GCV but no bacteria showed a small reduction in tumor volume that was noticeable within 5 days of GCV treatment and which persisted through the duration of the experiment, as shown in Figure

11C-E.   GCV also elicited small but reproducible increases in
survival time compared to that of non-treated control
animals, as outlined in Table 19(A). These effects of GCV in
the absence of bacterial treatment were not dependent upon
5 dosage over the range studied.

These results demonstrate that the B16F10 cells
employed in the experiment might have had the capacity to
convert GCV to its toxic, phosphorylated form. Consistent
with such a notion, it was found that proliferation of the
10 B16F10 melanoma cells in culture was significantly suppressed
when GCV was supplemented to the culture medium at 25 μg/ml,
but not at 10 μg/ml as shown in Figure 11-F. Similar effects
of GCV on DBA/2J mice bearing Cloudman S91 melanoma x
macrophage hybrid 48, but not inoculated with bacteria, are
15 reported in the Table 19.

2)   Effects of GCV on tumor-bearing animals treated
with bacteria not containing an HSV TK-plasmid

When tumor-bearing mice were inoculated with
20 _Salmonella_ strains YS7211, YS7212 and YS7213, none of which
contained the _HSV_ TK gene, and then treated with GVC, GCV-
mediated suppression of tumor growth was evident. Tumor
suppression achieved with GCV was significantly greater than
that seen with the bacteria alone, even when the suppressive
25 effect of GCV on B16F10 tumors in control animals was taken
into account. This indicated that _Salmonella typhimurium_
could convert GCV to its phosphorylated, toxic form without
the _HSV_ TK gene, perhaps through endogenous
phosphotransferase enzymes (Littler, et al., 1992, Nature
30 358:160-162; Sullivan et al., 1992, Nature 358:362-364).
Consistent with this notion was the finding that in addition
to suppressing tumor growth, some combinations of bacteria
and GCV treatment were highly toxic, shortening survival
times of the animals, shown in Table 19(A). Toxicity might
35 have resulted from production of phosphorylated GCV by those
bacteria located in normal tissues such as liver or bone
marrow.

3)   Effects of GCV on tumor-bearing animals treated
        <u>with bacteria containing an HSV TK-plasmid</u>

          Tumor-bearing mice inoculated with *HSV* TK plasmid-
containing *Salmonella* clone YS7211 (YS7211/p5-3) showed
5 suppression of tumor growth and prolonged survival even in
the absence of GCV treatment as shown in Figure 11-G.
Further, animals bearing both tumors and YS7211/p5-3 and
additionally treated with 3.75mg GCV showed significant
suppression of tumor growth above that seen in the absence of
10 GCV.   Using YS7211/p5-3 as a vector, GCV-mediated tumor
suppression was evident in a dose-responsive manner when
measured 28 days post implantation of tumors as shown in
Figure 11-H.   Tumor suppression correlated with increased
average survival times for some categories of GCV-treated,
15 tumor-bearing mice when compared to those inoculated with
YS7211/p5-3 but not receiving GCV.

          In summary:
          1)   In tumor-bearing animals not inoculated with
20 *Salmonella*, GCV had a small suppressive effect on tumor
growth that correlated with a small prolongation of survival.
          2)   Tumor-bearing animals inoculated with *Salmonella*
not containing the *HSV* TK plasmid showed marked tumor
suppression in response to GCV, above that seen in animals
25 not treated with bacteria.   In addition, some combinations of
GCV and bacterial treatment were highly toxic to the animals,
possibly through conversion of GCV to its toxic form by
bacteria in extra-tumoral tissues such as liver or bone
marrow.
30   3)   Tumor-bearing animals inoculated with *Salmonella*
containing the *HSV* TK plasmid also showed strong tumor
suppression in response to GCV.   It was not possible in these
experiments to evaluate the relative contributions of *HSV* TK
as compared to endogenous *Salmonella* enzymes in the
35 phosphorylation of GCV.   However, using as a vector clone
YS7211 containing the *HSV* TK expression plasmid, GCV-mediated

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tumor suppression and prolonged survival was demonstrated in a dose-dependent manner, see Figure 11-H.

17.   EXAMPLE: LOCALIZATION OF *SALMONELLA TYPHIMURIUM* WITHIN HUMAN TUMORS GROWN IN *NU/NU* MICE

The following experiments demonstrate localization of *Salmonella* in human tumors in experimental animals.

17.1.   LOCALIZATION OF *SALMONELLA* WITHIN HUMAN COLON TUMORS

NU/NU (BALB C) mice (9-10 weeks old) were inoculated s.c. in two areas (shoulder and flank), each with 1.5×10^7 HCT 116 human colon carcinoma cells. After the appearance of palpable, vascularized tumors (approximately 2 weeks) the animals were further inoculated i.p. with 3×10^5 *Salmonella typhimurium* super-infective clone 72^{5-3-2} carrying the *HSV* thymidine kinase gene. After 3.5 hours, 21 hours, and 72 hours of infection, mice were euthanized by anesthesia with metofane. Tumors and livers were removed aseptically, rinsed with sterile NaCl (0.9%), weighed, and homogenized with LB broth at a ration of 5:1 (vol. broth:wt. tumor). At 72 hours, prior to homogenization, pieces (1-2 mm^3) were removed from representative tumors, fixed with 1/2 strength Karnovsky's fixative, and processed for analysis with the electron microscope. Bacteria in the homogenates were quantitated by plating onto LB plates, incubating overnight at 37°C, and counting bacterial colonies.

Results were as follows: At 3.5 hours and 21 hours there were insignificant levels of bacteria in the tumors or livers, even when the homogenates were plated undiluted onto LB agar plates. However, after 3 days 3/6 animals displayed high levels of *Salmonella* in the colon tumors, with bacterial tumor:liver ratios ranging up to 36,000:1. Data for these animals are summarized below in Table 20.

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TABLE 20

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* 3 DAYS FOLLOWING
INOCULATION (I.P.) INTO HUMAN COLON CARCINOMA-BEARING *NU/NU*
MICE

Mouse A	*Salmonella/* gm tissue	Tumor/ Liver
Liver	2.6×10^4	
Tumor	6.9×10^8	26,500:1
Mouse D		
Liver	1.6×10^6	
Tumor	3.1×10^9	2,000:1
Mouse E		
Liver	1.0×10^5	
Tumor	3.6×10^9	36,000:1

        Shown in Figure 12-A an electron micrograph of a
section from the HCT colon tumor excised from mouse A (Table
20) in which the number of *Salmonella* found to be 6.9×10^8/g
tumor, and the tumor:liver ratio of infecting bacteria was
26,500:1. Shown in the micrograph are numerous *Salmonella
typhimurium* within a vacuole in the cytoplasm of a neutrophil
associated with the tumor. Some of the bacteria are
undergoing division as denoted by the arrow. The neutrophil
or polymorphonucleoleukocyte is characterized by its multi--
lobed nucleus (n). *Salmonella* in tumor-associated
neutrophils was also seen in infected B16F10 melanomas as
described herein. The presence of bacteria in both colon and
melanoma tumor-associated neutrophils following infection of
tumor-bearing mice suggests that the *Salmonella* may have
stimulated a host cellular immune response to the tumor
cells. Enhancement of tumor immunity is thus another
potential advantage in the use of parasites as tumor-specific
therapeutic vectors.

17.2.   **LOCALIZATION OF *SALMONELLA* WITHIN VARIOUS HUMAN TUMORS**

Nu/nu (BALB C) mice (9-12 weeks old) were
inoculated s.c. in the left shoulder region with 1-1.5 x 10⁷
5   cells of the human lung carcinoma A549, human colon carcinoma
HCT 116, human renal carcinoma CRL 1611, or human hepatoma
HTB 52 (American Type Culture Collection). When palpable
tumors developed, the mice were inoculated further with 2-5 x
10⁶ cfu *Salmonella typhimurium* clone 72 for animals bearing
10 human lung, liver, and renal tumors, and clone 72⁵⁻³⁻² for
animals bearing human colon tumors. Clone 72⁵⁻³⁻² carries the
HSV thymidine kinase transcription unit. After 66-96 hours
the animals were sacrificed, and the tumors and livers were
removed and weighed. The tumor was homogenized in 5 vol LB
15 broth/gram wet weight tissue. Homogenates were quantitated
by serial dilution on LB agar plates for the number of
bacteria. The results are presented in Table 20(A) and
represent the average ± standard deviation for n=3-4 animals.

20   TABLE 20(A)

BIODISTRIBUTION OF *SALMONELLA TYPHIMURIUM* CLONE 72 IN *NU/NU*
MICE BEARING HUMAN CARCINOMAS OF THE LUNG, COLON, KIDNEY,
AND LIVER

Salmonella/g tissue:				
Primary Tumor	Tumor	Liver	Tumor wt (mg)	Tumor: Liver
lung carcinoma	3.2 ± 1.4 x 10⁹	1.0 ± 0.3 x 10⁷	462 ± 186	320:1
colon carcinoma	2.5 ± 1.6 x 10⁹	5.8 ± 8.9 x 10⁵	428 ± 235	4300:1
hepatoma	6.7 ± 11 x 10⁸	5.7 ± 9.0 x 10⁶	103 ± 29	120:1
renal carcinoma	1.4 ± 1.8 x 10⁸	6.0 ± 3.0 x 10⁵	103 ± 99	230:1

35   As shown in Table 20-A, when inoculated i.p. into
nu/nu mice, *Salmonella typhimurium* clone 72 was able to

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target human carcinomas of the lung, colon, kidney, and liver, and proliferate within them, generally, but not always, reaching levels of 10^8-10^9/g tumor. In the BALB/c *nu/nu* mice used, the skin was hairless and translucent

5 allowing it to be determined visually that all the tumors were vascularized. The ranges of wet weights of the *Salmonella*-infected tumors were lung carcinoma, 220-600 mg; colon carcinoma, 160-600 mg; hepatoma, 70-120 mg; and renal carcinoma, 40-250 mg.

10 Bacterial colonies were picked randomly from liver and tumor homogenates obtained from renal carcinoma- and hepatoma-bearing *nu/nu* mice 96 hrs post-inoculation of clone 72 and tested for phenotype by replicate plating. In all homogenates tested, 50/50 colonies were found to be Ade⁻ and

15 Xyl^{neg}, consistent with the clone 72 phenotype.

The results further support the notion that derivatives of *Salmonella typhimurium* are useful as therapeutic vectors for a broad range of solid tumors, independent of the tumor origin or size. In several studies

20 *Salmonella* clone 72 and its derivatives targeted and amplified within highly vascularized tumors as small as 40-100 mg in the case of human tumors in *nu/nu* mice, as well tumors of 4-8 g with large necrotic areas in the case of B16F10 melanomas in C57B6 mice. The ability to target and

25 amplify within small vascularized tumors presents a distinct advantage of *Salmonella typhimurium* as a therapeutic tumor vector.

30 17.3. **LOCALIZATION BY ELECTRON MICROSCOPY OF SALMONELLA TYPHIMURIUM WITHIN HUMAN LUNG CARCINOMA A549**

A mouse was inoculated s.c. in the left shoulder region with 5×10^6 A549 cells. After 6 weeks the tumor was palpable and the animal was inoculated i.p. with 3×10^6 *Salmonella typhimurium* clone 72, for 66 hours. The animal

35 was sacrificed and a portion of the tumor was homogenized and found to contain 1.6×10^9 *Salmonella typhimurium*/g. The

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central portion of the tumor was prepared for electron
microscopy as follows: The portion of the tumor was cut into
1-2 mm³ pieces and fixed in 1/2 strength Karnovsky's fixative
for 6 hours at 4°C, followed by washing in cacodylate buffer
5   overnight. The tumor tissue was post-fixed with 1% OsO_4 and
1.5% potassium ferrocyanide in cacodylate buffer for 2 hours
and embedded in Spurr's resin. Ultrathin sections were
stained with uranyl acetate and lead citrate. They were
photographed through a Zeiss 109 electron microscope. It
10 should be noted for comparison purposes that the *S.
typhimurium* shown in the electron micrograph of Figure 12-B
appear similar to those shown previously in intestinal
epithelial cells following an experimental infection of the
mouse, Takeuchi, 1967, Am. J. Pathol. 50:109-1361.
15   Shown in Figure 12-B are numerous *Salmonella
typhimurium*, denoted by arrows, in extracellular spaces as
well as contained within a single cell, possibly a
neutrophil, seen in the upper left. Also seen in the field
are two unidentified cells that appear to be dying as
20 indicated by the large intracellular space, along with
cellular debris.

18. **EXAMPLE: ATTENUATION OF *SALMONELLA TYPHIMURIUM* BY
     MUTATION TO AUXOTROPHY**

25   The studies below demonstrate that the reduced
virulence of clone 72 (see, *e.g.*, Section 15.2 above) is due
to a Pur⁻ phenotype. Further described are analyses of
avirulent derivatives of clone 72 that were isolated as
additional auxotrophic mutants, expressing in different
30 combinations the phenotypes of Ade⁻, Ilv⁻, Arg⁻, Aro⁻, and
Ura⁻.

18.1.   **MUTATION TO AUXOTROPHY**

          Clone 72 was examined for auxotrophic mutations and
35 was found to have growth requirements for both adenine and
vitamin B1, indicating a mutation(s) in the purine
biosynthetic pathway (Pur⁻). An experiment was designed to

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test whether the ade⁻ mutation could account for the observed
attenuation of clone 72 described above. Populations of both
wild type strain 14028 and clone 72 were mutagenized with UV
radiation and nitrosoguanidine as described in Section 7.1.
5 From the population of mutagenized strain 14028, three
separate Pur⁻ auxotrophic mutant clones were isolated and
designated clones N, Q, and T. From the population of
mutagenized clone 72, three separate Pur⁺ revertant clones
were isolated and designated clones R, U, and W.
10   C57B/6 mice were injected i.p. with 2 x 10⁶ c.f.u.
Salmonella typhimurium of each of the strains obtained. The
mice were allowed to eat and drink *ad libitum* and the cages
were monitored for dead or moribund mice. Moribund animals
(listless, cessation of drinking) were euthanized and counted
15 with the other dead. After 10 or 30 days post-injection with
bacteria the surviving animals were euthanized.
         The results are shown in Table 20(B).

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TABLE 20(B)

SURVIVAL OF C57B6 MICE INJECTED WITH DIFFERENT AUXOTROPHIC
MUTANTS OF *SALMONELLA TYPHIMURIUM*

Strain	Phenotype	Time of Death (Days ± S.D.)	Survivors > 10 days	Survivors > 30 days
14028	wild type	3.0 ± 0.5	n.a.	n.a.
72	superinfective, ade⁻	5.8 ± 1.4	n.a.	n.a.
R	72, Pur⁺	3.9 ± 0.4	n.a.	n.a.
U	72, Pur⁺	3.9 ± 1.3	n.a.	n.a.
W	72, Pur⁺	4.1 ± 0.9	n.a.	n.a.
T	14028, Pur⁻	6.8 ± 1.5	n.a.	n.a.
N	14028, Pur⁻	n.a.	4/8	n.d.
Q	14028, Pur⁻	n.a.	5/8	n.d.
YS721	72, Ilv⁻	n.a.	10/11	6/11
YS7211	72, Ilv⁻, Arg⁻	n.a.	8/8	7/8
YS7213	72, Ilv⁻, Aro⁻	n.a.	8/8	8/8
YS7212	72, Ilv⁻, Ura⁻	n.a.	8/8	6/8

Results are the average ± SD for n = 8-12 animals
n.a., not applicable; n.d., not done.

As shown in Table 20(B), Clone 72 was less virulent
than the wild type strain 14028. However, 3 of 3 Pur⁺
revertants of clone 72 (U, W, and T) expressed virulence
similar to 14028. Conversely, 3 of 3 Pur⁻ auxotrophic mutants
isolated from strain 14028 (T, N, and Q) were less virulent
than either 14028 or clone 72.

Isolation of additional auxotrophs from clone 72 produced even less virulent strains. For example, clone YS721 is an isoleucine-valine requiring (Ilv⁻) derivative of clone 72, and clone YS721 was significantly less virulent

5   than clone 72. Similarly, auxotrophic derivatives of clone YS721 such as clones YS7211 (Arg⁻), YS7212 (Ura⁻), and YS7213 (Aro⁻) were all significantly less virulent than YS721 itself.

18.2. **EVIDENCE THAT THE SUPERINFECTIVE**
10   **PHENOTYPE OF CLONE 72 IS GENETICALLY**
             DISTINCT FROM ITS AUXOTROPHIC PURINE
             REQUIREMENT

The various *Salmonella typhimurium* Pur⁻ and Pur⁺ strains described above in Section 18.1 were assayed for their ability to infect human M2 melanoma cells in culture.

15 The *in vitro* infection assay employed was as described in Section 18.1.

The results are described in Table 20(C).

TABLE 20(C)

20

INFECTIVITY TOWARD HUMAN M2 MELANOMA CELL *IN VITRO* BY VARIOUS PURINE MUTANTS OF *SALMONELLA TYPHIMURIUM*

Strain	Phenotype	Infecting *Salmonella*/ 10^6 melanoma cells/15'($\pm$S.D.)	x wild type
14028	wild type	$1.0 \pm 0.2 \times 10^5$	1.0x
72	superinfective, ade⁻	$9.8 \pm 0.7 \times 10^5$	9.8x
R	72, Pur⁺	$5.9 \pm 1.4 \times 10^5$	5.9x
U	72, Pur⁺	$1.1 \pm 0.2 \times 10^6$	11x
W	72, Pur⁺	$1.1 \pm 0.3 \times 10^6$	11x
N	14028, Pur⁻	$1.9 \pm 0.5 \times 10^5$	1.9x
Q	14028, Pur⁻	$1.5 \pm 1.0 \times 10^5$	1.5x
T	14028, Pur⁻	$1.1 \pm 0.4 \times 10^5$	1.5x

Results are the average ± SD for triplicate infections. The bacteria were cultured in LB broth to O.D.$_{600}$ = .600 prior to their dilution and use in the infection assays.

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As shown in Table 20(C), Clone 72 displayed superinfectivity toward human M2 melanoma cells compared to wild type strain 14028. None of the 14028 Pur⁻ derivatives differed significantly in its infectivity from strain 14028

5 itself, and all of the clone 72 Pur⁺ derivatives expressed superinfectivity similar to clone 72 itself. The results demonstrate that the purine requirement exhibited by clone 72 which accounts for the reduced virulence of clone 72 in mice, is genetically separate from the superinfective phenotype of

10 clone 72. These results demonstrate that neither mutation to nor reversion from purine auxotrophy effects expression of the superinfective phenotype characteristic of clone 72.

        18.3.   **RETENTION OF THE SUPERINFECTIVE PHENOTYPE BY ATTENUATED DERIVATIVES OF** *SALMONELLA*

15   *TYPHIMURIUM* **CLONE 72**

In the experiments below, the infectivity of certain auxotrophic derivatives of clone 72 described above in Section 18.1 was assessed *in vitro*. The phenotypes of the clones of *Salmonella* evaluated are shown in Table 20(B) in

20 Section 18.1 above. Infectivity assays described in Section 10.1 were employed.

The results are presented in Table 20(D).

TABLE 20(D)		
INFECTIVITY OF *SALMONELLA TYPHIMURIUM* **AUXOTROPHS TOWARD HUMAN MELANOMA CELLS IN CULTURE**		
<u>Strain</u>	Infecting *Salmonella*/ 10^6 melanoma cells/15 min	<u>x wild type</u>
14028 (wild type)	$4.3 \pm \times 10^4$	1.0x
clone 72	$4.4 \pm \times 10^5$	10x
clone YS721	$3.2 \pm \times 10^5$	7.4x
clone YS7211	$2.0 \pm \times 10^5$	4.7x
clone YS7212	$1.7 \pm \times 10^5$	4.0x
clone YS7213	$1.3 \pm \times 10^3$	0.03x

35 The results represent the average ± SD for 10-19 separate infections. The bacteria were grown in LB broth to O.D.$_{600}$ = 0.5 before being diluted prior to their use in the infection assays

Salmonella typhimurium clones YS721, YS7211, and
YS7212, though each somewhat less infective of M2 melanoma
cells than clone 72, were nonetheless superinfective when
compared to wild type strain 14028, indicating their partial
5 retention of the superinfective phenotype. In contrast,
clone YS7213 (Ade⁻, Ilv⁻, Aro⁻) was found to have greatly
reduced infectivity, being about 30-fold less infectious
toward M2 melanoma cells than the wild type strain 14028.

10   18.4.   **GROWTH OF PUR⁻ AND URA⁻ MUTANTS OF**
                       ***SALMONELLA TYPHIMURIUM* WITH NUTRITIONAL**
                       ADDITIVES OR EXTRACTS OF B16F10 MELANOMA

Tumor extracts were prepared in the following
manner: B16F10 melanoma tumor cells (5×10^5) were implanted
s.c. into 68 week female C57B6 mice. After 3-4 weeks, the
15 mice were sacrificed and the tumors removed aseptically and
rapidly frozen, -20°C. A total of 51 g of frozen pooled
tumors was thawed at 4°C and vigorously homogenized in 255 ml
(5 vol) H_2O in a capped Virtis tissue homogenizer in the cold
for 1 hour. The resulting homogenate was made 10% with
20 trichloracetic acid (TCA), placed on ice for 15 minutes, and
centrifuged in a Beckman J21 centrifuge at about 20,000x g
for 15 minutes at 4°C. Further procedures were conduced at
room temperature. The clear, colorless supernatant fraction
(300 ml) was retained and extracted by manual shaking for
25 1 minute with 1 volume (300 ml) anhydrous ether. Between
extractions, the mixtures were allowed to settle and the
upper phase (containing ether, extracted TCA, as well as
ether-soluble compounds from the tumor extract) was removed
by aspiration and discarded through approved environmentally-
30 protective procedures. During 5 such extraction cycles, the
pH of the water phase rose from a starting value of about
pH 1 to a final value of pH 4-5, similar to that of distilled
H_2O, indicating that the TCA had been effectively removed.
The water phase was bubbled with a stream of nitrogen for
35 about 15 minutes, at which time the odor of ether had
disappeared.

The solution was then filtered through a 0.2 micron filter, divided into aliquots and either used directly in the assays herein, or stored at -20°C for further use.

5   Wild type strain 14028, and its auxotrophic derivatives clone 72 (Pur⁻, vitamin B1⁻), and YS7212 (Ade⁻, vitamin B1⁻, Ilv⁻, Ura⁻) were grown overnight on a slant in 5 ml Luria broth (LB) at 35°C. The next day 0.1 ml of each culture was diluted into 10 ml of Medium 56 (0.037 M KH_2PO_4, 0.06 M Na_2HPO_4, 0.02% $MgSO_4-7H_2O$, 0.2% $(NH_4)_2SO_4$, 0.001% $Ca(NO_3)_2$

10 and 0.00005% $FeSO_4-7H_2O$) supplemented with 0.2 µg/ml vitamin B1, 33 µg/ml adenine, 50 µg/ml uracil, 83 µg/ml isoleucine, 83 µg/ml valine and 0.3% glucose, and grown on a rotor overnight at 37°C. The next day the cultures were collected by centrifugation and resuspended in plain

15 Medium 56 (1 ml culture plus 9 ml Medium 56). Aliquots (0.25 ml) of these suspensions were then added to Medium 56 containing various supplements in the following manner:

A.   Medium 56 plus glucose;

B.   Medium 56 plus glucose, vitamin B1, adenine, isoleucine,
20   valine, and uracil; and

C.   Medium 56 and tumor extract (10%).

The bacteria were placed in a swirling H_2O bath, 37°C, and growth as a function of OD_{600} was followed with a spectrophotometer. The starting optical densities for all of

25 the cultures ranged from 0.005-0.07.

As demonstrated in Figure 15A-C, wild type strain 14028 was able to proliferate at about the same rates in all three of the media tested, including the most basic of the three, Medium 56 plus glucose. Unlike the wild type strain,

30 neither clone 72 nor clone YS7212 was able to grow in Medium 56 plus glucose, indicative of their nutritional requirements originally observed through replicated plating on agar. In contrast both clone 72 and clone YS7212 were able to grow in Medium 56 supplemented with 10% tumor extract. Liver

35 extracts prepared in the same manner were also able to support the growth of clones 72 and YS7212.

Although the inventors do not wish to be limited to a specific mechanism of action, since the growth state of auxotrophic strains of *Salmonella* is dependent upon the availability of nutrients, such auxotrophs would seem to have
5 advantages as tumor vectors since the environment of the tumor could in theory provide such nutrients, for example in necrotic spaces or within actively dividing cells of the tumor. Thus, mutation of organisms such as *Salmonella* to auxotrophy not only reduces their virulence *in vivo* but also
10 may provide a potential mechanism for their selective population and amplification within solid tumors.

18.5.   **PROLIFERATION OF PUR⁻ AND URA⁻ MUTANTS OF *SALMONELLA TYPHIMURIUM* IN HUMAN M2 MELANOMA CELLS IN CULTURE**

15   In this Section it is demonstrated that the internal milieu of cultured M2 melanoma cells also can also supply the auxotrophic requirements of these clones, since both clone 72 and clone YS7212 were able to undergo several rounds of division once they invaded M2 melanoma cells
20 cultured under aerobic conditions.

Salmonella typhimurium clones 72 and YS7212 were grown to $O.D._{600}=0.8$, or about 10^9 c.f.u./ml. The two strains were then added at 10^6 c.f.u./ml culture media of human M2 melanoma cells as described above in Section 7.2. 15 minutes
25 after infection with *Salmonella*, the eukaryotic cell cultures were rinsed with fresh medium and medium containing gentamicin (10 μg/ml) was added. At hourly intervals over a 6 hour period, cultures were processed as described in Section 7.2 for quantitation of *Salmonella*/10^6 melanoma cells.
30 In addition, control flasks without melanoma cells but with bacteria were processed side-by-side with the experimental flasks containing melanoma cells.

The results are shown in Figure 15-D. Control flasks with *Salmonella* but without melanoma cells showed no
35 viable bacteria over the 6 hour period, demonstrating that the wash procedure coupled with gentamicin treatment

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successfully eliminated all viable bacteria not protected by
location within animal cells. In contrast, in the presence
of M2 melanoma cells, *Salmonella typhimurium* clones 72 and
YS7212 each increased significantly in number over the 6 hour
5 period with doubling times of about 2 hours for each strain.
Phase and electron microscope analyses demonstrated that M2
melanoma cells were able to compartmentalize infecting
Salmonella within vacuoles. The results indicate that the
net rate of growth of *Salmonella* within the melanoma cells
10 was a steady-state function, reflecting the ability of the
melanoma cells to both stimulate growth of the auxotrophs
through the supply of nutritional requirements, as well as to
suppress the growth of the auxotrophs through anti-bacterial
mechanisms.

15

18.6.   **BIODISTRIBUTION OF AUTOTROPHIC ATTENUATED
          STRAINS OF *SALMONELLA TYPHIMURIUM* IN
          C57B6 MICE-BEARING B16 MELANOMA TUMORS**

These studies demonstrate the ability of clones
YS721, YS7213, YS7211 and YS7212 to target tumors and
20 proliferate within the tumor *in vivo*.

C57B6 6-8 week old female mice were inoculated s.c.
(left flank) with 2.5-5.0 x 10^5 B16F10 mouse melanoma cells.
When the tumors reached about 0.5 g (14-16 days post-tumor
inoculum), the animals were further inoculated i.p. with the
25 indicated strains of *S. typhimurium*. The bacterial inoculum
was 4 x 10^5 cfu/mouse for strains 14028 and 72 and 2-4 x 10^6
cfu/mouse for strains YS721, YS7211, YS7212 and YS7213.
After 40 and 96 hours post-inoculation of bacteria, the mice
were sacrificed, the tumors and livers were removed
30 aseptically, rinsed with sterile NaCl (0.9%), weighed, and
homogenized in LB broth at a ratio of 5:1 (vol:tumor wt).
Bacteria were quantitated by plating the homogenates onto LB
plates, incubating overnight at 37°C, and counting bacterial
colonies. The results presented in Table 20(E) represent the
35 average ± SD for n=4-7 animals.

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TABLE 20(E)

BIODISTRIBUTION OF WILD TYPE AND ATTENUATED STRAINS OF
SALMONELLA TYPHIMURIUM IN C57B6 MICE-BEARING B16 MELANOMA
TUMORS

	Salmonella/g tissue:		
Strain	Tumor	Liver	Tumor: Liver
A. 40 hrs post-inoculation of bacteria			
14028	$6.5 \pm 6.8 \times 10^9$	$2.4 \pm 2.8 \times 10^7$	270:1
72	$1.7 \pm 1.2 \times 10^9$	$1.9 \pm 2.3 \times 10^5$	9000:1
YS721	$8.7 \pm 3.1 \times 10^8$	$4.2 \pm 3.6 \times 10^6$	210:1
YS7211	$3.3 \pm 3.0 \times 10^7$	$8.1 \pm 8.4 \times 10^5$	41:1
YS7212	$3.9 \pm 7.3 \times 10^7$	$1.1 \pm 0.8 \times 10^6$	35:1
YS7213	$1.5 \pm 2.8 \times 10^8$	$4.0 \pm 3.1 \times 10^5$	375:1
B. 96 hrs post-inoculation with bacteria			
14028	moribund/dead		
72	moribund/dead		
YS721	$3.2 \pm 1.5 \times 10^9$	$4.7 \pm 6.9 \times 10^6$	680:1
YS7211	$1.6 \pm 2.2 \times 10^9$	$6.3 \pm 9.9 \times 10^6$	253:1
YS7212	$1.1 \pm 7.4 \times 10^9$	$5.1 \pm 8.6 \times 10^5$	2200:1
YS7213	$1.3 \pm 2.5 \times 10^9$	$2.2 \pm 6.9 \times 10^5$	5900:1

Each of the strains tested was able to target the
tumor tissue and replicate to varying degrees within the
tumor, as evidenced by the finding that in all cases the
tumors contained 10-1000 times more *Salmonella typhimurium*
than were first inoculated. Further, in all cases the
tumor:liver ratio of bacteria/g tissue was at least 35:1 and
in some cases approached 10^4. The tumors analyzed in the
studies presented in Table 20(E) ranged in weights from 0.5-
2.0 g. Of all the conditions and strains tested, clone 72
exhibited the highest tumor:liver ratio 40 hours post
inoculation. Further, *Salmonella typhimurium* strain 14028,
as well as clone 72 and its derivatives were also able to
target and amplify within larger B16F10 melanoma tumors of,
for example, 4-8 g. In addition, as shown in Section 10.3.2

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and Table 12A, clone 72 can target and amplify within human
solid tumors as small as 40 mg.

However, both clone 72 and the wild type strain
14028 were highly virulent toward C57B6 mice, especially mice
5 bearing tumors. For example, C57B/6 mice bearing B16F10
melanomas injected with strains 1428 and 72 had average
survival times of 2.1 ± 0.4 days (n=6) and 4.7 ± 0.5 days
(n=9) post-inoculation of bacteria respectively. The
biodistribution of these strains was thus not measured at
10 96 hours. Likewise, clone YS721, though attenuated compared
to 14028 and clone 72, was nonetheless virulent in melanoma-
bearing mice. For example, B16F10 melanoma-bearing C57B6
mice injected with clone YS721 had an average survival time
of 8.1 ± 0.2 days (n=11). _Salmonella_ clones YS7211, YS7212
15 and YS7213, the least virulent of those examined, each
displayed densities of greater than 10^9 cfu bacteria/g tumor
96 hrs post-inoculation with tumor:liver ratios of 253:1,
2200:1, and 5900:1 respectively.

20   18.7.   **PHENOTYPIC STABILITY FOLLOWING INCUBATION
                       OF _SALMONELLA TYPHIMURIUM_ AUXOTROPHS IN
                       TUMOR-BEARING MICE**

Genetic reversion of an auxotrophic phenotype could
in theory result in an increase in virulence of the
previously attenuated bacteria. Therefore, the stabilities
25 of the auxotrophic phenotypes of the strains YS7211, YS7212
and YS7213 were tested following incubation of the bacteria
in tumor-bearing mice.

Salmonella typhimurium obtained from the
homogenates of livers and tumors of animals 40 hours post-
30 inoculation of either YS7211, YS7212 or YS7213 were picked
from LB plates and replicate plated onto minimal media agar
plates supplemented with nutritional additives in different
combinations. The supplements were isoleucine, valine,
adenine/vitamin B1, arginine, uracil, aromatic amino acids,
35 and glucose. For each of the three strains, 50/50 of the
bacterial clones recovered from the tumor and liver

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homogenates displayed the expected phenotypes of the strain originally inoculated, indicating that in this experiment the strains were genetically stable enough not to revert substantially under the conditions tested.

5   However, it should be noted that the auxotrophic strains employed were not absolutely stable throughout these studies. In some cases genetic revertants were observed, most notably in the YS7211 strain wherein revertants from Arg⁻ to Arg⁺ were observed. For example, in a tumor-bearing mouse

10 inoculated 96 hours earlier with clone YS7211 bearing a thymidine kinase-containing plasmid, 50 out of 50 bacteria isolated from the liver were found to be Pur⁻, Ilv⁻ and Arg⁺, indicating that reversion and selective growth of the reverted organisms had occurred within the mouse. The

15 finding that the auxotrophic phenotypes of clones YS7211, YS7212 and YS7213 were relatively stable in mice was supported by the long term survival of mice inoculated with these strains.

20   18.8.   **SUPPRESSION OF TUMOR GROWTH AND INCREASED SURVIVAL OF C57B6 TUMOR-BEARING MICE INOCULATED WITH AUXOTROPHIC MUTANTS OF _SALMONELLA TYPHIMURIUM_**

C57B6 female mice, 5-7 weeks old, were inoculated s.c. in the left shoulder region with 5×10^5 B16F10 melanoma

25 cells grown in culture. On the 8th day following inoculation of tumor cells, the mice were further inoculated i.p. with $2-4 \times 10^6$ c.f.u. of _Salmonella typhimurium_ strains YS721, YS7211, YS7212 or YS7213. Tumor growth was assessed with periodic caliper measurements of tumor length, width and

30 height, and computed as tumor volume in mm^3. Results of tumor growth, shown in Figure 16A-D represent the averages ± SD for 5 animals/group with 5/5 animals surviving. After the point at which one or more animals died within a group, the average tumor sizes of the surviving animals were no longer shown

35 when the data were plotted as shown in Figure 16A-D.

All tumor measurements were stopped after 33 days post implantation of tumor cells, even though 5/5 tumor-

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bearing animals treated with clone YS7211 were still alive at
this time.   The animals were allowed to eat and drink *ad
libitum*.   Twenty-three days (Experiment #1) or 10 days
(Experiment #2) following inoculation of bacteria, both .
5 control and bacteria treated mice were given Baytril™
(enrofloxacin, 0.2 mg/ml drinking water) and maintained on
this antibiotic for a total of 2 weeks.   In Experiment #1 the
times at which the mice became moribund (listless, cessation
of drinking) or died, were noted.   The results are presented
10 in Table 20(F) as the average survival ± SD for the
conditions tested.   In Experiment #2 animals were sacrificed
when the tumor reached 4 g and listed with the other dead as
described in Experiment #1.   The two different methods for
assessing survival accounted for a somewhat shorter survival
15 time for control animals in Experiment #2 (26 days) as
compared to Experiment #1 (28 days).

TABLE 20(F)			
SURVIVAL OF B16F10 MELANOMA-BEARING C57B6 MICE INOCULATED WITH *SALMONELLA TYPHIMURIUM*			

Strain		Time of death post tumor cell inoculation: (Days ± SD)	Treated/Control (T/C)
Control (no bacteria)	Expt 1	28 ± 2	1.0
	2	26 ± 3	1.0
YS7211	1	36 ± 9	1.3
	2	41 ± 10	1.6
YS7213	1	36 ± 5	1.3
	2	38 ± 6	1.5
YS7212	1	51 ± 7	1.8
	2	55 ± 3	2.1

35 The results represent the average ± SD for 5 animals.

Figure 16A-D shows the average ± SD tumor volumes (mm^3) versus time post inoculation of 5×10^5 B16F10 melanoma cells s.c. into C57B/6 mice. All four clones of *Salmonella*, namely clones YS721, YS7211, YS7212 and YS7213, elicited

5   suppression of tumor growth in the animals. Clone YS721, attenuated through Ade⁻ and Ilv⁻ auxotrophy, was nonetheless toxic to tumor-bearing mice and resulted in no prolongation of survival compared to control tumor-bearing animals receiving no bacteria. Whereas the death of control animals

10   was clearly due to very large tumor masses (4-8 g), the death of tumor-bearing animals inoculated with clone YS721 appeared to be a result of bacterial toxicity since the tumor burden in these animals was quite small and not life-threatening in itself. The tumors ranged from non-palpable to less than

15   0.5 g at the time of death.

In contrast, treatment of tumor-bearing mice with clone YS7211, YS7212 and YS7213, each less virulent than clone YS721, resulted in significant enhancement of survival in addition to suppression of tumor growth. The degree of

20   suppression of tumor growth by the individual *Salmonella* clones, as seen in Figure 16A-D, correlated with their abilities to elicit enhanced survival, as seen in Table 20(F). The average time for tumors to reach 1 g (1000 mm^3) was about 18 days for control animals, 31 days for animals

25   treated either with YS7213 and YS7211 and 45 days (extrapolated) for YS7212. This corresponded to average survival times for 26 days for control tumor-bearing animals, compared to 38, 41, and 55 days for animals treated with clones YS7213, YS7211 and YS7212.

30   Thus, among the attenuated strain of *Salmonella* tested, the order of efficacy for suppression of tumor growth and prolongation of survival was YS7212>YS7211>YS7213. Earlier treatment with an antibiotic, enrofloxacin, *i.e.*, 10 days as compared to 23 days post-inoculation of bacteria,

35   increased the survival time for tumor-bearing animals inoculated with *Salmonella*, but not that of control animals.

18.9.   ANTI-TUMOR ACTIVITY OF AUXOTROPHIC
          SALMONELLA TYPHIMURIUM EXPRESSING
          CYTOSINE DEAMINASE

Experimental metastasis model of B16F10 was set up
by injecting 1 x 10⁵ cells into C57B/6 mice via the lateral
tail vein on Day 0. Aliquots of 0.2 ml bacterial suspension
of YS7212 carrying the cytosine deaminase expression
construct (see Figure 4E for the CD construct) (approximately
1 x 10⁷ CFU/ml) were injected intraperitoneally into mice on
Day 5. 5-Flourocytosine (5-FC), at 0.4 ml aliquots dissolved
in PBS at 10 mg/ml (final dose: 200 mg/kg), was injected into
mice intraperitoneally on Day 7. Death of animals was
recorded daily. Results are presented in Figure 17.

Figure 17 clearly demonstrates that combination of
CD and 5-fluorocytosine prolong the survival *Salmonella*
expressing animals bearing B16F10 lung metastases.

19.   **EXAMPLE: ATTENUATION OF *SALMONELLA TYPHIMURIUM***
      THROUGH MUTATION IN LIPOPOLYSACCHARIDE
      BIOSYNTHESIS

Several mutant strains of *Salmonella typhimurium*
and *E. coli* have been isolated with genetic and enzymatic
lesions in the LPS pathway (Raetz, 1993, J. Bacteriol.
175:5745-5753). One such mutant, the *firA⁻* mutation is within
the gene that encodes the enzyme UDP-3-O(R-30
hydroxymyristoyl)-glycocyamine N-acyltransferase, that
regulates the third step in endotoxin biosynthesis (Kelley et
al., 1993, J. Biol. Chem. 268:19866-19874). *Salmonella
typhimurium* and *E. coli* strains bearing this type of mutation
produce a lipid A that differs from wild type lipid A in that
it contains a seventh fatty acid, a hexadecanoic acid (Roy
and Coleman, 1994, J. Bacteriol. 176:1639-1646) and has
decreased lipid A 4' kinase activity.

A *firA⁻* mutant was investigated for its ability to
induce TNFα production by human monocytes as well as its
ability to target solid tumors in mice.

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19.1.   ABILITY OF *SALMONELLA TYPHIMURIUM firA⁻* TO
INDUCE TNF-α PRODUCTION BY HUMAN BLOOD
MONOCYTES

Salmonella typhimurium strain SH5014 and its *firA⁻*
5 derivative SH7622 are described in Hirvas et al., 1991, EMBO
J. 10:1017-1023. The genotypes of these strains are as
follows:

strain SH5014 *ilv*-1178 *thr*-914 *his*-6116 *metA22 metE551 trpB2*
xyl-404 *Hl-b H2-e,n,x flaA66 rpsL120 rfaJ4041;*

10 **strain SH7622** *ilv*-1178 *thr*-914 *his*-6116 *metA22 metE551 trpB2*
xyl-404 *Hl-b H2-e,n,x flaA66 rpsL120 rfaJ4041, ssc-1(firA^{ts}).*

A derivative of *Salmonella typhimurium firA⁻* strain
SH7622 was picked, designated SH7622-64, and used as the *firA⁻*
strain for the experiments in this section as well as in
15 Section 19.2 below. SH7622-64 was selected for its
supersensitivity to the antibiotic novobiocin and
temperature-sensitive growth, characteristics of the *firA⁻*
SH7622 strain.

LPS was extracted from *Salmonella typhimurium*
20 strain 14028 and its derivatives clone 72, clone YS7212, and
clone YS7213; as well as strain SH5014 and its *firA⁻*
derivative, clone SH7622-64, as follows: The bacteria were
grown in 500 ml LB broth to O.D.$_{600}$=0.9 or about 2×10^9
cfu/ml. They were collected by centrifugation, and the
25 pellets, containing about 10^9 cfu/ml. They were collected by
centrifugation, and the pellets, containing 10^{12} bacteria,
were drained and stored frozen at -20°C. To extract LPS, the
pellets were resuspended in 18.3 ml H$_2$O, and 15 ml redistilled
phenol was added (H$_2$O:phenol, 55:45, vol/vol). The mixtures
30 were placed in a shaking water bath at 69-70°C, for 1 hour
producing a monophasic mixture, and then cooled on ice. On
cooling the mixture separated into a phenol phase containing
mainly proteins, and a water phase containing
lipopolysaccharide and nucleic acid (Galanos, C., Luderitz,
35 O., and Westphal, O., 1969). The water phase was lyophilized
to dryness and the white fluffy lyophilized material was used
as the source of LPS. The LPS was weighed and dissolved in

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H_2O at 1 mg/ml, as stock for dilution in the incubations with
human macrophages described below.

Human macrophages were prepared as follows and all
procedures were at room temperature: Blood (60 ml) was
5   collected from a healthy human volunteer into a heparinized
syringe. The blood was layered in 7 ml aliquots over 4 ml of
Isolymph™ (density - 1.077 g/ml; 9.0 g sodium diatrizoate and
5.7 g Ficoll 400™/100 ml H_2O; Pharmacia Fine Chemicals, A.B.
Uppsala, Sweden) in 15 ml Corning Plastic Centrifuge tubes,
10 centrifuged at 2000x g for 45 minutes. The red blood cells
pelleted through the Isolymph™, neutrophils and other cells
sedimented in a discrete band above this interface, and above
the lymphocyte/macrophage band was serum, visible by its
yellow color. The serum from each tube was removed by
15 pipette, pooled in a total volume of about 30 ml and saved
for supplementation into the culture media as described
below. The lymphocyte/macrophage bands were pooled in a
total volume of about 15 ml diluted with 40 ml RPMI 1640
culture medium, and centrifuged at 1000x g for 5 minutes.
20 The cloudy supernatant was discarded and about 0.2 ml of
pelleted white cells was obtained. The cells were
resuspended with 50 ml RPMI 1640 culture medium supplemented
with 15% human serum (described above), penicillin (100
units/ml) and streptomycin (100 μg/ml). The recovery of
25 viable lymphocytes and macrophages from 60 ml whole blood was
determined by hemocytometer counting to be about 7 x 10^7
cells. Together the cells, lymphocytes and monocytes were
distributed into 24 well Corning Tissue Culture Plates at
0.5 ml/well, and incubated in a gassed humidified incubator
30 at 37°C for 15 hours.

The next day the cultures were rinsed twice with
serum-free RPMI 1640 containing antibiotics. Between each
rinse, the cultures were incubated about 1 hour in the 37°C
incubator to facilitate removal of lymphocytes and other non-
35 adherent cells. Adherent cells were found to be mostly, if
not all derived from blood monocytes, i.e., macrophages that
had differentiated from their blood monocyte state by virtue

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of attachment to the culture dish. For example, in a
histochemical assay to determine the percentage of
macrophages in the adherent population of cells 48 hours
post-plating into culture, the population was found to be
5 essentially 100% positive for expression of the enzyme non-
specific esterase, a marker commonly used to distinguish
monocytes and macrophages from lymphocytes and other cell
types. Results indicated that most if not all of the cells
employed in the LPS challenge described below were of
10 monocyte origin.

After the second rinse described above, serum-free,
antibiotic containing RPMI 1640 supplemented with LPS at the
concentrations indicated was added to the cells, and the
cultures were placed in a gassed, humidified incubator at
15 37°C overnight. After 20 hours, the well plate cultures were
centrifuged in a Beckman GS-15 centrifuge at 8000x g for 10
minutes, and the supernatants were removed and assayed for
TNF-α content using the QUANTIKINE™ Human TNF-α Immunoassay
Kit #DTA50 (R&D Systems, Minneapolis, MN). TNF-α production
20 as pg/ml by human macrophages is plotted as a function of
pg/ml bacterial LPS added to the culture medium and shown in
Figure 18.

Strain 14028 and its derivatives clone 72, clone
YS7212, and clone YS7213, as well as strain SH5014, all
25 induced TNF-α production by human macrophages at
concentrations of LPS in a dose-dependent fashion.
Concentrations of LPS from each of these strains as low as
100 pg/ml (0.1 ng/ml) were stimulatory to TNF-α production,
and increasingly stimulatory at concentrations of 10^3 pg/ml
30 and 10^4 pg/ml, inducing TNF-α production by macrophages to
levels of 600-800 pg/ml. The levels of TNF-α induced by the
LPS were similar to the circulating levels of TNF-α found in
patients with septic shock syndrome as well as in human
volunteers injected with E. coli LPS (Morrison et al., 1994,
35 ASM News 60:479-484). In contrast, LPS from firA⁻ strain
SH7622-64 was far less stimulatory to TNF-α production by the
macrophages, and was detected only at concentrations of

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10^4 pg/ml. Thus, on a dose response comparison, LPS from strain SH7622-64 was only about 1% as effective in stimulating macrophage TNF-α production when compared to LPS from the *firA*$^+$ parental strain SH5014. Furthermore, the

5 results demonstrate that strains YS7212 and YS7213 each produced LPS similar to wild type strain 14028 LPS, as assessed by stimulation of human macrophages.

19.2. **TUMOR TARGETING BY *SALMONELLA TYPHIMURIUM*
BEARING THE *firA*$^-$ MUTATION**

10

M27 mouse lung tumor cells or B16F10 mouse melanoma cells (5 x 10^5) were implanted s.c. in C57B6 mice. When the tumors were palpable, SH7622-64 grown in LB broth at 37°C to a density of about 10^9 cfu/ml (OD$_{600}$=0.8). Aliquots of 5-10 x

15 10^6 cfu were removed and inoculated into tumor bearing mice. At 48 hrs (M27 lung) and 96 hrs (B16F10 melanoma) post-inoculation of bacteria the animals were sacrificed, the tumors and livers removed, weighed and homogenized in LB broth at a ratio of 5 ml/g tissue. Homogenates were

20 quantitated for bacteria by serial dilutions on LB agar plates. Results are presented in Table 20(G).

TABLE 20(G)

---

25 TUMOR LOCATION BY *SALMONELLA TYPHIMURIUM* BEARING THE *firA*$^-$ MUTATION FOR LIPOPOLYSACCHARIDE BIOSYNTHESIS

Primary Tumor	*Salmonella/g* tissue:		
	Tumor	Liver	Tumor: Liver
M27 lung	2.9×10^6	-0-	n.a.
B16	3.2×10^5	1×10^2	3200:1

The results are derived from single animals.

35

As shown in Table 20(G), strain SH7622-64 was able to locate within both the B16F10 melanoma and the M27 lung tumors when inoculated i.p. into mice. These results, in combination with those in Section 19.1 which show that LPS from this particular *firA⁻* strain was greatly suppressed in its ability to induce TNF-α in human macrophages, demonstrate that *Salmonella* attenuated through a mutation in endotoxin biosynthesis can be useful as tumor vectors *in vivo*.

20. **EXAMPLE: TUMOR-SPECIFIC ACCUMULATION OF CLONES YS721 AND YS7211 IN MURINE LEWIS LUNG CARCINOMA**

This example demonstrates that auxotrophic mutant *Salmonella* clones YS721 and YS7211 locate to lung carcinoma.

The experimental model of the Lewis lung carcinoma was set up by injecting 5×10^5 cells into C57B/6 mice subcutaneously on Day 0. Aliquots of 0.2 ml bacterial suspension (approximately 1×10^7 CFU/ml) were injected intraperitoneally into mice on Day 14. On Day 16, the tumors and livers were harvested and homogenized and bacterial counts determined by plating serial dilutions. Results of the relative distribution are shown in Table 20(H).

TABLE 20(H)			
TUMOR SPECIFIC ACCUMULATION OF CLONES YS721 and YS7211 IN MICE			
Strain	No. pathogens /g Liver	No. pathogens /g Tumor	Ratio: tumor/liver
YS721	9.8×10^6	4.7×10^{10}	4.8×10^3
	4.3×10^5	3.2×10^{10}	7.3×10^4
	1.1×10^6	1.4×10^9	1.3×10^3
	1.6×10^6	1.0×10^{12}	6.2×10^5
	3.0×10^4	2.3×10^9	7.7×10^4
YS7211	1.4×10^4	2.6×10^{10}	1.9×10^6
	1.9×10^5	2.7×10^8	1.4×10^3
	2.3×10^5	6.0×10^{11}	2.6×10^6
	1.0×10^6	5.0×10^{11}	5.0×10^5

Extremely high levels of bacteria were localized to
these tumors, as well as others indicating that the
auxotrophic mutations retain tumor specific accumulation of
bacteria for a spectrum of tumor models.

21. EXAMPLE: TREATMENT OF B16F10 MELANOMA METASTATIC TUMORS

Metastases constitute one of the major problems for
treatment of solid tumors. While larger tumors can be
detected and removed surgically, smaller metastases
constitute the untreated reservoir which is frequently the
cause of death. Therefore, an effective cancer therapeutic
should be effective against metastatic tumors.

An experimental metastasis model of B16F10 was set
up by injecting 1×10^5 CELLS into C57B/6 mice via the lateral
tail vein on Day 0. Aliquots of 0.2 ml YS7211/p5-3 and
YS7212/p5-3 (YS7211 and YS7212 each carrying the *HSV*
thymidine kinase expression plasmid) bacterial suspensions
(approximately 1×10^7 CFU/ml) were injected intraperitoneally
into mice on Day 5. Ganciclovir, at 0.1 ML aliquots

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dissolved in PBS at 22 mg/ml (final dose: 100 mg/kg), was
injected into mice intraperitoneally on Day 7. Tumor
progression was monitored by periodic sacrifice and
examination of the lungs. At day 28, all the animals were
5 sacrificed and the normal and tumor-bearing lungs weighted.

Figure 19 clearly demonstrates that animals
inoculated with YS7212 carrying the *HSV* thymidine kinase gene
(YS7212/p5-3) and further treated with GCV show reductions in
the number and extent of B16F10 lung metastases.

10

22. EXAMPLE: DIAGNOSIS OF TISSUE BIOPSIES FOR MELANOMA USING *SALMONELLA TYPHIMURIUM*

Diagnosis of melanoma according to the methods of
the present invention can be performed using, for example,
15 *Salmonella typhimurium* as follows: A portion of a biopsied
specimen suspected of melanoma is minced with scissors in
tris-buffered saline (TBS) and then incubated in Ca^{++}/Mg^{++} -
free saline containing trypsin, collagenase, and EDTA (Sigma
Chemicals) for 60 minutes at 37°C to dissociate the tissue
20 into individual cells. The cells are then rinsed free of the
dissociation enzymes by centrifugation. The cells are
resuspended in 1 ml DMEM/10% FBS and added to a 24 well
Corning tissue culture chamber containing cover slips in the
wells. The cells are then incubated in a gassed (5% CO_2/95%
25 air) humidified incubator for 3 hours at 37°C to allow for
attachment to the cover slip.

After attachment of the biopsied cells is achieved,
an attenuated, super-infective, melanoma-specific strain of
Salmonella typhimurium (10^6-10^7 c.f.u./ml) is added. The
30 bacteria and biopsied cells are incubated together at 37°C
for 15 minutes to allow for infection of melanoma cells by
the *S. typhimurium*, and the cells are then rinsed with TBS to
remove non-infecting bacteria. The cells are then
permeablized with 0.01% saponin in 3% bovine serum albumin
35 for 5 min, stained for DNA for 10 minutes with 2.5 mg/ml 4'-6
Diamidino-2-phenyhndole (DAPI) and saponin (.01%) in TBS,
washed with TBS, mounted in Mowiol (Calbiochem) containing 1,

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4-Diazabicyclo (2,2,2) octane (DABCO, Kodak) and observed by phase and fluorescence microscopy. The presence of DAPI-stain in the cytoplasm of the biopsied cells would indicate that they were melanoma cells, *i.e.*, cells that were infected

5 by the melanoma-specific *S. typhimurium* are melanoma cells rather than melanocytes.

23. EXAMPLE: MELANOMA TUMOR TARGETING BY *LISTERIA MONOCYTOGENES*

10 This example demonstrates that *Listeria monocytogenes* targets to and proliferates in tumor cells when administered to melanoma bearing animals.

C57B/6 mice were inoculated s.c. in the left flank with 5×10^5 B16F10 melanoma cells. When the tumors reached

15 about 1-2 g (16 days post implantation of tumor cells) the animals were inoculated i.p. with 7×10^5 cfu of *Listeria monocytogenes* wild type strain 43251. Prior to inoculation into mice, the *Listeria* culture was grown overnight in LB media to an OD_{600} of 0.25. At the times indicated animals

20 were sacrificed and the tumors and livers were removed, homogenized and quantitated for bacterial numbers by plating serial dilutions onto L.B. plates.

Tumors were analyzed at 24, 48, and 96 hours post-inoculation of *Listeria monocytogenes*. Results are shown in

25 Table 20(I).

30

35

TABLE 20(I)

AMPLIFICATION OF *LISTERIA MONOCYTOGENES* IN C57B/6 MICE-BEARING B16F10 MELANOMA TUMORS

Time Post-inoc.	*L. monocytogenes*/g tissue:		
	Tumor	Liver	Tumor: Liver
24 hrs	$1.5 \pm 1.4 \times 10^3$	$8.0 \pm 6.1 \times 10^4$	1:5
48 hrs	$6.3 \pm 8.5 \times 10^2$	$1.3 \pm 1.0 \times 10^5$	1:210
96 hrs	$5.2 \pm 6.8 \times 10^5$	$5.7 \pm 9.0 \times 10^5$	1:1

The results represent the average ± SD of triplicate determinations.

As shown in Table 20(I) it was found that the levels of bacteria within the tumors rose about 100 fold during this time period, indicating that wild type *Listeria monocytogenes* can target tumors and proliferate within them. *Listeria monocytogenes* strain 43251 was virulent in the C57B/6 mice, causing death about 5 days pot-inoculation i.p. of 7×10^5 cfu.

24. **EXAMPLE: *LEISHMANIA AMAZONENSIS* SHOWS TUMOR CELL SPECIFICITY**

   24.1.   ***LEISHMANIA AMAZONENSIS* SPECIFICALLY ATTACHES TO HUMAN MELANOMA CELLS *IN VITRO***

Leishmania amazonensis trypomastigotes are regarded as being highly biospecific, in that they are unable to infect virtually any cell types other than macrophages. Since human melanomas are known to express some macrophage-like traits it was determined whether *Leishmania amazonensis* would be able to enter into human melanoma cells in culture. *Leishmania amazonensis* promastigotes were grown in Schneider's Drosophila media (GIBCO BRL) containing 15% heat-inactivated fetal calf serum at 24°C until the parasites were in late log phase (usually 3 to 4 days). Animal cells used

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in the *L. amazonensis* infection assays were a mouse melanoma
cell line which forms non-metastatic tumors when injected
into C57B6 mice (B16/F1), two human metastatic melanoma cell
lines (M2 and M2-A7, and as a negative control human foreskin
5 fibroblasts, HFF. These different cell types were grown on
glass coverslips in 24 well plates or on plastic Lab-Tec®
(Nunc) slides in MEM culture medium with 10% fetal calf
serum, for HFF cells; Ham's F10 medium with 10% horse serum
for B16/F1 cells; and DMEM with 10% fetal calf serum buffered
10 with 10 mM HEPES, for M2 and M2-A7 cells.

        The *Leishmania* parasites were pre-incubated for
about one hour with 5% normal human serum and the cultured
cells were infected with 0.5 to 5.0×10^6 parasites/ml for about
two hours at 32°C. After incubation the cells were washed
15 twice with phosphate buffered saline (PBS) and fixed with 3%
paraformaldehyde for about 30 minutes at about 4°C. An anti-
Leishmanial antibody was incubated with the fixed cells at a
normal working dilution (1: 100,000) in PBS with 3% bovine
serum albumen (BSA) for about one hour. After washing, a
20 fluorescent-conjugated anti-mouse antibody (Boehringer
Mannheim) was incubated with the cells at a normal working
dilution (1:500) for about one hour and then washed from the
cells. The cells were then permeabilized with 0.02% Saponin
(Sigma, a detergent used to remove lipids, thereby allowing
25 penetration by antibodies) in Tris buffered saline (TBS) for
10 min and stained for DNA with 5.0 mg/ml DAPI stain (Sigma)
in TBS with 0.02% Saponin. The cells were washed with TBS
and mounted on glass slides using Mowiol (CalBiochem) with
DABCO (Kodak, a compound that sustains fluorescent
30 emissions). The presence of internalized parasites was
determined by failure to react with an anti-*Leishmania*
monoclonal antibody in the absence of host cell membrane
permeabilization.

        Observations made immediately after addition of the
35 live parasites using an inverted phase microscope showed that
among these cell lines, motile parasites were immediately
adherent only when they encountered the metastatic melanoma

cells, suggesting that the metastatic cells may possess an appropriate receptor for *Leishmania*. These results are shown in Figure 13.

5 To determine whether or not the *Leishmania* parasites were internalized, M2 human melanoma cells were grown, infected with *Leishmania* and fixed as described above with 3% paraformaldehyde at 4°C for about 30 minutes without permeabilization, washed and immunostained with a monoclonal antibody directed toward a *Leishmania* surface protein,

10 followed by a rhodamine-conjugated anti-mouse antibody. After washing in Tris-buffered saline (TBS), the cells were permeablized with 0.01% saponin in 3% BSA for 5 minutes, and stained for DNA 10 minutes with 2.5 mg/ml 4'-6 Diamidino-2-phenylindole (DAPI) and 0.01% saponin in TBS, washed with

15 TBS, mounted in Mowiol (Calbiochem) containing 1, 4-Diazabicyclo (2,2,2) octane (DABCO, Kodak) and observed by phase and fluorescence microscopy. This procedure detects all parasites and distinguishes between those which are internalized (inaccessible to antibody staining in non-

20 permeabilized cells), and those which are attached but not internalized.

Parasites were internalized by M2 cells (data not shown). Internalization was estimated to occur in 3% of the melanoma cells. These findings demonstrate that a) live

25 *Leishmania* parasites were able to enter the melanoma cells, and b) possibly only a sub-population of the melanoma cells were involved in the process.

24.2.   **LYSOSOMAL FUSION FOLLOWS INTERNALIZATION**
30   **OF *LEISHMANIA* BY MELANOMA**

In the normal course of invasion of macrophages by *Leishmania*, lysosomes fuse with the phagosome. To determine whether or not this also occurs when *Leishmania* invade melanoma cells, parasites were co-localized with a lysosomal

35 glycoprotein (lgp) marker. The cells were grown, infected and fixed as described above except the cells were immunostained with a monoclonal antibody directed against a

human lysosomal glycoprotein, LAMP-1, followed by a
rhodamine-conjugated antimouse antibody. After washing in
TBS, the cells were stained for DNA 10 minutes with 2.5 mg/ml
DAPI, washed with TBS, mounted in Mowiol containing DABCO and
5 observed by phase and fluorescence microscopy. Parasites co-
localizing with LAMP-1 are shown in Figures 14A-C. Co-
localization corroborates the internalization of the parasite
and demonstrates that the process of lysosomal fusion occurs
when *Leishmania* is internalized into the melanoma cells. In
10 summary, *Leishmania amazonensis* in its wild type state shows
invasion ability for human melanoma cells that has been
heretofore unreported.

    25. **EXAMPLE: DIAGNOSIS OF MELANOMA IN HUMAN TISSUE
          BIOPSIES USING *LEISHMANIA AMAZONENSIS***
15
         Diagnosis of melanoma according to the methods of
the present invention can be performed using, for example,
Leishmaina Amazonensis as follows: A portion of a biopsied
specimen suspected of melanoma is minced with scissors in
20 tris-buffered saline (TBS) and then incubated in Ca^{++}/Mg^{++}-free
saline containing trypsin, collagenase, and EDTA at 37°C for
60 minutes to dissociate the tissue into individual cells.
The cells are then rinsed free of the dissociation enzymes by
centrifugation. The cells are resuspended in 1 ml DMEM/10%
25 FBS and added to a 24 well Corning tissue culture chamber
containing cover slips in the wells. The cells are then
incubated in a gassed, 5% CO_2, humidified incubator at 37°C
for about three hours to allow for attachment to the cover
slip.
30   After attachment of the biopsied cells is achieved,
a melanoma-specific strain of *Leishmania amazonensis*
promastigotes which has been isolated according to the
methods of the present invention is added. The parasites and
biopsied cells are incubated together at 37°C for about two
35 hours to allow for infection of melanoma cells by the
Leishmania amazonensis and the cells are then rinsed with TBS
to remove non-infecting parasites. The cells are then

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permeablized with 0.01% saponin in 3% bovine serum albumin
for five minutes, stained for DNA with 2.5 mg/ml 4'-6
Diamiclino-2-phenylindole (DAPI) and saponin (.01%) in TBS
for 10 minutes, washed with TBS, mounted in Mowiol
5 (Calbiochem) containing 1,4-Diazabicyclo (2,2,2) octane
(DABCO, Kodak) and observed by phase and fluorescence
microscopy. The presence of DAPI-stain in the cytoplasm of
the biopsied cells would indicate that they were melanoma
cells.

10

26. EXAMPLE: DIAGNOSIS OF HUMAN TISSUE BIOPSIES FOR MELANOMA USING *MYCOBACTERIUM AVIUM*

Mycobacterium avium were found associated with the
melanoma cells but not with the normal melanocytes. This
discriminatory ability for melanoma cells demonstrates the
15 ability of *Mycobacterium avium* as a vector in the diagnosis
and treatment of melanoma. Diagnosis of melanoma using
Mycobacterium avium is as follows: A portion of a biopsied
specimen suspected of melanoma is minced with scissors in
tris-buffered saline (TBS) and then incubated in Ca^{++}/Mg^{++}-free
20 saline containing trypsin, collagenase, and EDTA at 37°C for
60 minutes to dissociate the tissue into individual cells.
The cells are then rinsed free of the dissociation enzymes by
centrifugation. The cells are resuspended in 1 ml DMEM/10%
FBS and plated onto 12mm glass cover slips in 24 well plates
25 with $1x10^5$ cells per well. The cells are then incubated in a
gassed 5% CO_2, humidified incubator 37°C for 3 hours to allow
for attachment of the cells to the cover slip.

After attachment of the biopsied cells is achieved,
a melanoma-specific strain of *Mycobacterium avium* which has
30 been isolated by the methods of the present invention is
added. The bacteria and biopsied cells are incubated
together at 37°C for 15 minutes for infection of melanoma
cells by the *Mycobacterium avium*. The cells are then rinsed
with TBS to remove non-infecting bacteria. The cells are
35 then permeablized with 0.01% saponin in 3% bovine serum
albumin for five minutes, stained for DNA with 2.5 mg/ml 4'-6

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Diamidino-2-phenylindole (DAPI) and saponin (.01%) in TBS for 10 minutes, washed with TBS, mounted in Mowiol (Calbiochem) containing 1, 4-Diazabicyclo (2,2,2) octane (DABCO, Kodak) and observed by phase and fluorescence microscopy. The
5 presence of DAPI-stain in the cytoplasm of the biopsied cells would indicate that they were melanoma cells.

27. DEPOSIT OF MICROORGANISMS

The following microorganisms were deposited with
10 the American Type Culture Collection (ATCC), Rockville, MD on June 1, 1995 and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.
Clone #70	55686
Clone #71	55685
Clone #72	55680
Clone #72^{5-3-2}	97179
Population #72^{pop-1}	55684
Population #72^{pop-2}	55683
Population #14028^{pop-1}	55681
Population #14028^{pop-2}	55682

The following microorganisms were deposited with the American Type Culture Collection (ATCC), Rockville, MD on
25 May 29, 1996, and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.
Clone YS721	_____
Clone YS7211	_____
Clone YS7212	_____
Clone YS7213	

The following plasmids were deposited with the American Type Culture Collection (ATCC), Rockville, MD on May
35 29, 1996, and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.

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pTK-Sec3   _____
pCD-Sec1   _____
pSP-SAD4-5   _____

5   The invention claimed and described herein is not
to be limited in scope by the specific embodiments herein
disclosed since these embodiments are intended as
illustrations of several aspects of the invention. Indeed,
various modifications of the invention in addition to those
10 shown and described herein will become apparent to those
skilled in the art from the foregoing description. Such
modifications are also intended to fall within the scope of
the appended claims.
        A number of references are cited herein, the
15 entire disclosures of which are incorporated herein, in their
entirety, by reference.

20

25

30

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Pawelek, John M.
              Bermudes, David
              Low, Kenneth Brooks

5

    (ii) TITLE OF INVENTION: VECTORS FOR THE DIAGNOSIS AND TREATMENT
           OF SOLID TUMORS INCLUDING MELANOMA

   (iii) NUMBER OF SEQUENCES: 10

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        (B) STREET: 1155 Avenue of the Americas

10   (C) CITY: New York
        (D) STATE: New York
        (E) COUNTRY: U.S.A.
        (F) ZIP: 10036-2711

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS

15   (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: To Be Assigned
        (B) FILING DATE: On Even Date Herewith
        (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Baldwin, Geraldine F.
        (B) REGISTRATION NUMBER: 31,232

20   (C) REFERENCE/DOCKET NUMBER: 8002-036

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (212) 790-9090
        (B) TELEFAX: (212) 869-9741/8864
        (C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

25

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

30

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATCATGCAT GGCTTCGTAC CCCGGCC   27

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
35   (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

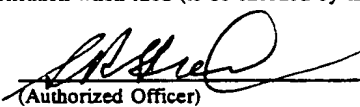5   CTAGATGCAT CAGTGGCTAT GGCAGGGC   28

    (2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 31 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

10   (ii) MOLECULE TYPE: DNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

    CTAGACTAGT TTGTCAATAA TGACAACACC C   31

15 (2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

20

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

    GATCGGATCC TTGCCCGGCG CGGCGGCCTG   30

    (2) INFORMATION FOR SEQ ID NO:5:

25   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 32 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

30
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

    CTAGAAGCTT ATAAGGGTTG ATCTTTGTTG TC   32

    (2) INFORMATION FOR SEQ ID NO:6:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 31 base pairs
35   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

5 GTACGATATC CAGAACGATG TGCATAGCCT G   31

(2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 9 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: peptide

10

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

        Tyr Thr Ser Gly Tyr Ala His Arg Ser
        1   5

15 (2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 6 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: peptide

20

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

        Ser Gly Tyr Arg Ile Pro
        1   5

(2) INFORMATION FOR SEQ ID NO:9:

25   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 25 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

30

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCATGCAT GTGGAGGCTA ACAGT   25

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
35   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

5   CTAGATGCAT CAGACAGCCG CTGCGAAGGC   30

10

15

20

25

30

35

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 139-140, lines 1-35 of the description '

A. IDENTIFICATION OF DEPOSIT '

Further deposits are identified on an additional sheet '

Name of depositary institution '

American Type Culture Collection

Address of depositary institution (including postal code and country) '

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit ' June 1, 1995 Accession Number ' 55686

B. ADDITIONAL INDICATIONS ' (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ' (If the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS ' (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later ' (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the International application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau ·

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/   /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

Accession No.	Date of Deposit
55685	June 1, 1995
55680	June 1, 1995
97179	June 1, 1995
55684	June 1, 1995
55683	June 1, 1995
55681	June 1, 1995
55682	June 1, 1995
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996
	May 29, 1996

WHAT IS CLAIMED IS:

      1.   A biologically pure culture of microorganisms selected from the following strains:

Microorganism	ATCC Accession No.
Salmonella typhimurium strain #70	55686
Salmonella typhimurium strain #71	55685
Salmonella typhimurium strain #72	55680
Salmonella typhimurium strain #72^{5-3-2}	97179
Salmonella typhimurium strain #14028^{pop-1}	55684
Salmonella typhimurium strain #14028^{pop-2}	55683
Salmonella typhimurium strain #72^{pop-1}	55681
Salmonella typhimurium strain #72^{pop-2}	55682
Salmonella typhimurium strain YS721	_____
Salmonella typhimurium strain YS7211	_____
Salmonella typhimurium strain YS7212	_____ and
Salmonella typhimurium strain YS7213	_____ .

      2.   A plasmid encoding the Herpes Simplex Virus thymidine kinase gene designated pTK-Sec3 and having ATCC Accession No. _____.

      3.   A plasmid encoding the *E. coli* cytosine deaminase gene designated pCD-Sec1 and having ATCC Accession No. _____.

      4.   A plasmid encoding the human p450 oxidoreductase gene designated pSP-SAD4-5 and having ATCC Accession No. _____.

      5.   A method of treatment for a solid tumor cancer, comprising:
      administering an effective amount of an isolated
      population of tumor cell-specific microorganisms to a
      patient having a solid tumor cancer.

      6.   The method according to claim 5 wherein the isolated population of tumor cell-specific microorganisms is

an isolated population of super-infective, tumor cell-specific microorganisms.

7.   The method according to claim 6 wherein the super-infective, tumor cell-specific population is attenuated.

8.   The method according to claim 7 wherein the attenuated population expresses an altered lipid A molecule.

9.   The method according to claim 7 wherein the attenuated population induces TNF-α expression to a lesser degree compared to a non-attenuated population.

10.   The method according to claim 5 wherein the isolated population of tumor cell-specific microorganisms is selected from the group consisting of *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma hominis*, *Rickettsiae quintana*, *Streptococcus* spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp., *Trypanasoma cruzi*, *Encephahtozoon cuniculi*, *Nosema helminthorum* and *Unikaryon legeri*.

11.   The method according to claim 5 or 6 wherein the isolated population of super-infective, tumor cell specific microorganisms is selected from the group consisting of *Salmonella typhimurium* strain #14028^{POP-1}, *Salmonella*

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typhimurium strain #14028^{pop-2}, *Salmonella typhimurium* strain #72^{pop-1} and *Salmonella typhimurium* strain #72^{pop-2}.

12.   The method according to claim 6 wherein the
5 isolated population of super-infective, tumor cell specific microorganisms is produced by:
   (a) exposing a cell culture of a solid tumor cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells; and
10   (b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected cell culture.

13.   The method according to claim 5 wherein the
15 isolated population of tumor cell-specific microorganisms is produced by:
   (a) exposing a microorganism to tumor cell conditioned medium for a time sufficient to allow the microorganism to chemotact towards the tumor cell
20   conditioned medium; and
   (b) isolating a population of microorganisms which chemotacts towards the tumor cell conditioned medium.

25   14.   The method according to claim 6 wherein the isolated population of super-infective, tumor cell specific microorganisms is produced by:
   (a) exposing a mammal having a solid tumor cell cancer to a microorganism for a time sufficient so that
30   the microorganism can infect the tumor cells; and
   (b) isolating a population of super-infective, tumor cell-specific microorganisms from the infected tumor cells.

35   15.   The method according to claim 12, 13 or 14 further comprising:

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subjecting the microorganism to mutagenesis before
step (a).

16.   The method according to claim 12, 13 or 14
5 further comprising:
     (c)   repeating steps (a) and (b) a desired number of
            times.

17.   The method according to claim 5, 12, 13 or 14
10 wherein the solid tumor cell cancer is melanoma cancer.

18.   The method according to claim 5, 12, 13 or 14
wherein the solid tumor cancer is colon carcinoma cancer.

15   19.   The method according to claim 5, 12, 13 or 14
wherein the soild tumor cancer is selected from the group
consisting of lung cancer, liver cancer, kidney cancer,
prostate cancer and breast cancer.

20   20.   A method of treatment for a solid tumor
cancer, comprising:
     administering an effective amount of a single colony
     clone of an isolated population of tumor cell-specific
     microorganisms to a patient having a solid tumor cancer.
25

21.   The method according to claim 20 wherein the
single colony clone of an isolated population of tumor cell-
specific microorganisms is a single colony clone of an
isolated population of super-infective, tumor cell-specific
30 microorganisms.

22.   The method according to claim 20 wherein the
super-infective, tumor cell-specific single colony clone is
attenuated.
35

23.   The method according to claim 22 wherein the
attenuated clone expresses an altered lipid A molecule.

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24.   The method according to claim 20 wherein the attenuated clone induces TNF-α expression to a lesser degree compared to a non-attenuated clone.

5   25.   The method according to claim 20 wherein the single colony clone of an isolated population of tumor cell-specific microorganisms is selected from the group consisting of *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella*
10 *pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma hominis*, *Rickettsiae quintana*, *Streptococcus* spp.,
15 *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*, *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp.,
20 *Trypanasoma cruzi*, *Encephahtozoon cuniculi*, *Nosema helminthorum* and *Unikaryon legeri*.

26.   The method according to claim 20 or 21 wherein the single colony clone of an isolated population of super-
25 infective, tumor cell specific microorganisms is selected from the group consisting of *Salmonella typhimurium* strain #70, *Salmonella typhimurium* strain #71, *Salmonella typhimurium* strain #72, *Salmonella typhimurium* strain #72^{5-3-2}, *Salmonella typhimurium* strain YS721, *Salmonella typhimurium*
30 strain YS7211, *Salmonella typhimurium* strain YS7212 and *Salmonella typhimurium* strain YS7213.

27.   The method according to claim 21 wherein the single colony clone of an isolated population of super-
35 infective, tumor cell specific microorganisms is produced by:

(a)   exposing a cell culture of a solid tumor cancer to
       a microorganism for a time sufficient so that the
       microorganism can infect the tumor cells;

(b)   isolating a population of super-infective, tumor
5   cell-specific microorganisms from the infected cell
       culture; and

(c)   culturing the population isolated in step (b) so
       that single colony clones are obtained.

10   28.   The method according to claim 20 wherein the
single colony clone of an isolated population of tumor cell
specific microorganisms is produced by:

(a)   exposing a microorganism to tumor cell conditioned
       medium for a time sufficient to allow the
15   microorganism to chemotact towards the tumor cell
       conditioned medium;

(b)   isolating a population of microorganisms which
       chemotacts towards the tumor cell conditioned
       medium; and

20 (c)   culturing the population isolated in step (b) so
       that single colony clones are obtained.

           29.   The method according to claim 21 wherein the
single colony clone of an isolated population of super-
25 infective, tumor cell specific microorganisms is produced by:

(a)   exposing a mammal having a solid tumor cell cancer
       to a microorganism for a time sufficient so that
       the microorganism can infect the tumor cells;

(b)   isolating a population of super-infective, tumor
30   cell-specific microorganisms from the infected
       tumor cells; and

(c)   culturing the population isolated in step (b) so
       that single colony clones are obtained.

35   30.   The method according to claim 27, 28 or 29
further comprising:

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subjecting the microorganism to mutagenesis before step (a).

31. The method according to claim 27, 28 or 29 further comprising:

(d) repeating steps (a) and (b) a desired number of times before step (c).

32. The method according to claim 20, 27, 28 or 29 wherein the solid tumor cell cancer is melanoma cancer.

33. The method according to claim 20, 27, 28 or 29 wherein the solid tumor cancer is colon carcinoma cancer.

34. The method according to claim 20, 27, 28 or 29 wherein the soild tumor cancer is selected from the group consisting of lung cancer, liver cancer, kidney cancer, prostate cancer and breast cancer.

35. The method according to claim 20 or 21 wherein the single colony clone is genetically engineered.

36. The method according to claim 35 wherein the single colony clone expresses a suicide gene.

37. The method according to claim 36 wherein the suicide gene is encoded by a plasmid selected from the group consisting of pTK-Sec3, pCD-Sec1 and pSP-SAD4-5.

38. The method according to claim 36 wherein the single colony clone expresses a suicide gene selected from the group consisting of p450 oxidoreductase, *HSV* thymidine kinase, *E. coli* cytosine deaminase, carboxypeptidase G2, β-glucuronidase, penicillin-V-amidase, penicillin-G-amidase, β-lactamase, β-glucosidase, nitroreductase and carboxypeptidase A.

39. The method according to claim 37 or 38 wherein the suicide gene is selected from the group consisting of *HSV* thymidine kinase and *E. coli* cytosine deaminase.

5   40. The method according to claim 36 wherein expression of the suicide gene is controlled by a constitutive promoter.

41. The method according to claim 36 wherein
10 expression of the suicide gene is controlled by an inducible promoter.

42. The method according to claim 36 wherein expression of the suicide gene is controlled by a bacterial
15 promoter that is activated in specific target cells.

43. The method according to claim 42 wherein expression of the suicide gene is controlled by a bacterial promoter that is activated in specific tumor cells.
20

44. A method of diagnosis for a solid tumor cancer, comprising:

    (a)   exposing a biopsied sample of cells suspected of being cancer cells to a tumor-specific
25   microorganism for a time sufficient so that the microorganism can infect the cells suspected of being cancerous;

    (b)   exposing a sample of non-cancerous counterpart control cells to the tumor-specific microorganism
30   for the same time; and

    (c)   comparing the infectivity of the microorganism for the cells suspected of being cancerous and the non-cancerous counterpart control cells.

35   45. The method according to claim 44 wherein the microorganism is selected from the group consisting of *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*,

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enteroinvasive *Escherichia coli*, *Legionella pneumophila*,
Salmonella typhi, *Salmonella typhimurium*, *Shigella* spp.,
Treponema pallidum, *Yersinia enterocohtica*, *Chlamydia*
trachomatis, *Listeria monocytogenies*, *Mycobacterium avium*,
5 *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma*
hominis, *Rickettsiae quintana*, *Streptococcus* spp.,
Cryptococcus neoformans, *Histoplasma capsulatum*, *Pneumocystis*
carnii, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium*
falciparum, *Sarcocystis suihominis*, *Toxoplasma gondii*,
10 *Leishmania amazonensis*, *Leishmania major*, *Leishmania*
mexacana, *Leptomonas karyophilus*, *Phytomonas* spp.,
Trypanasoma cruzi, *Encephahtozoon cuniculi*, *Nosema*
helminthorum and *Unikaryon legeri*.

15   46.   The method according to claim 44 wherein the
microorganism is selected from the group consisting of
Salmonella typhimurium strain #14028^{POP-1}, *Salmonella*
typhimurium strain #14028^{POP-2}, *Salmonella typhimurium* strain
#72^{POP-1}, *Salmonella typhimurium* strain #72^{POP-2}, *Salmonella*
20 *typhimurium* strain #70, *Salmonella typhimurium* strain #71,
Salmonella typhimurium strain #72, *Salmonella typhimurium*
strain #72^{5-3-2}, *Salmonella typhimurium* strain YS721,
Salmonella typhimurium strain YS7211, *Salmonella typhimurium*
strain YS7212 and *Salmonella typhimurium* strain YS7213.
25

          47.   The method according to claim 44 wherein the
microorganism is produced by:
          (a)   exposing a cell culture of a solid tumor cancer to
                a microorganism for a time sufficient so that the
30   microorganism can infect the tumor cells; and
          (b)   isolating a population of super-infective, tumor
                cell-specific microorganisms from the infected cell
                culture.

35   48.   The method according to claim 44 wherein the
microorganism is produced by:

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(a)   exposing a microorganism to tumor cell conditioned medium for a time sufficient to allow the microorganism to chemotact towards the tumor cell conditioned medium; and

5   (b)   isolating a population of microorganisms which chemotacts towards the tumor cell conditioned medium.

49.   The method according to claim 44 wherein the
10 microorganism is produced by:

(a)   exposing a mammal having a solid tumor cell cancer to a microorganism for a time sufficient so that the microorganism can infect the tumor cells; and

(b)   isolating a population of super-infective, tumor
15   cell-specific microorganisms from the infected tumor cells.

50.   The method according to claim 47, 48 or 49 further comprising:

20   (c)   culturing the population isolated in step (b) so that single colony clones are obtained.

51.   The method according to claim 47, 48 or 49 further comprising:

25   subjecting the microorganism to mutagenesis before step (a).

52.   The method according to claim 47, 48 or 49 further comprising:

30   repeating steps (a) and (b) a desired number of times.

53.   The method according to claim 44, 47, 48 or 49 wherein the solid tumor cell cancer is melanoma cancer.

35

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54. The method according to claim 44, 47, 48 or 49 wherein the solid tumor cell cancer is colon carcinoma cancer.

5   55. The method according to claim 44, 47, 48 or 49 wherein the soild tumor cancer is selected from the group consisting of lung cancer, liver cancer, kidney cancer, prostate cancer and breast cancer.

10   56. The method according to claim 44 wherein the microorganism is genetically engineered.

57. A diagnostic kit comprising an effective amount of a tumor-specific microorganism.
15

58. The diagnostic kit according to claim 57 wherein the tumor-specific microorganism is a super-infective, tumor-specific microorganism.

20   59. The diagnostic kit according to claim 57 further comprising non-cancerous counterpart control cells.

60. The diagnostic kit according to claim 57 wherein the microorganism is selected from the group
25 consisting of *Borrelia burgdorferi*, *Brucella melitensis*, *Escherichia coli*, enteroinvasive *Escherichia coli*, *Legionella pneumophila*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella* spp., *Treponema pallidum*, *Yersinia enterocohtica*, *Chlamydia trachomatis*, *Listeria monocytogenies*, *Mycobacterium*
30 *avium*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycoplasma hominis*, *Rickettsiae quintana*, *Streptococcus* spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carnii*, *Eimeria acervulina*, *Neospora caninum*, *Plasmodium falciparum*, *Sarcocystis suihominis*, *Toxoplasma gondii*,
35 *Leishmania amazonensis*, *Leishmania major*, *Leishmania mexacana*, *Leptomonas karyophilus*, *Phytomonas* spp.,

Trypanasoma cruzi, *Encephahtozöon cuniculi*, *Nosema helminthorum* and *Unikaryon legeri*.

61.   The diagnostic kit according to claim 58
wherein the microorganism is selected from the group
consisting of *Salmonella typhimurium* strain #14028^{POP-1},
Salmonella typhimurium strain #14028^{POP-2}, *Salmonella typhimurium* strain #72^{POP-1}, *Salmonella typhimurium* strain #72^{POP-2}, *Salmonella typhimurium* strain #70, *Salmonella typhimurium* strain #71, *Salmonella typhimurium* strain #72, *Salmonella typhimurium* strain #72^{5-3-2} *Salmonella typhimurium* strain YS721, *Salmonella typhimurium* strain YS7211, *Salmonella typhimurium* strain YS7212 and *Salmonella typhimurium* strain YS7213.

62.   The diagnostic kit according to claim 58
wherein the microorganism is genetically engineered.

63.   The method according to claims 5, 12, 13 or 14
wherein the solid tumor cancer is a metastatic cancer.

64.   The method according to claims 20, 27, 28 or
29 wherein the solid tumor cancer is a metastatic cancer.

65.   The method according to claims 44, 47, 48 or
49 wherein the solid tumor cancer is a metastatic cancer.

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FIG. 1

FIG.2B

FIG.2A

FIG.3A

FIG.3B

FIG.3C

FIG.4A

FIG. 4B

FIG.4C
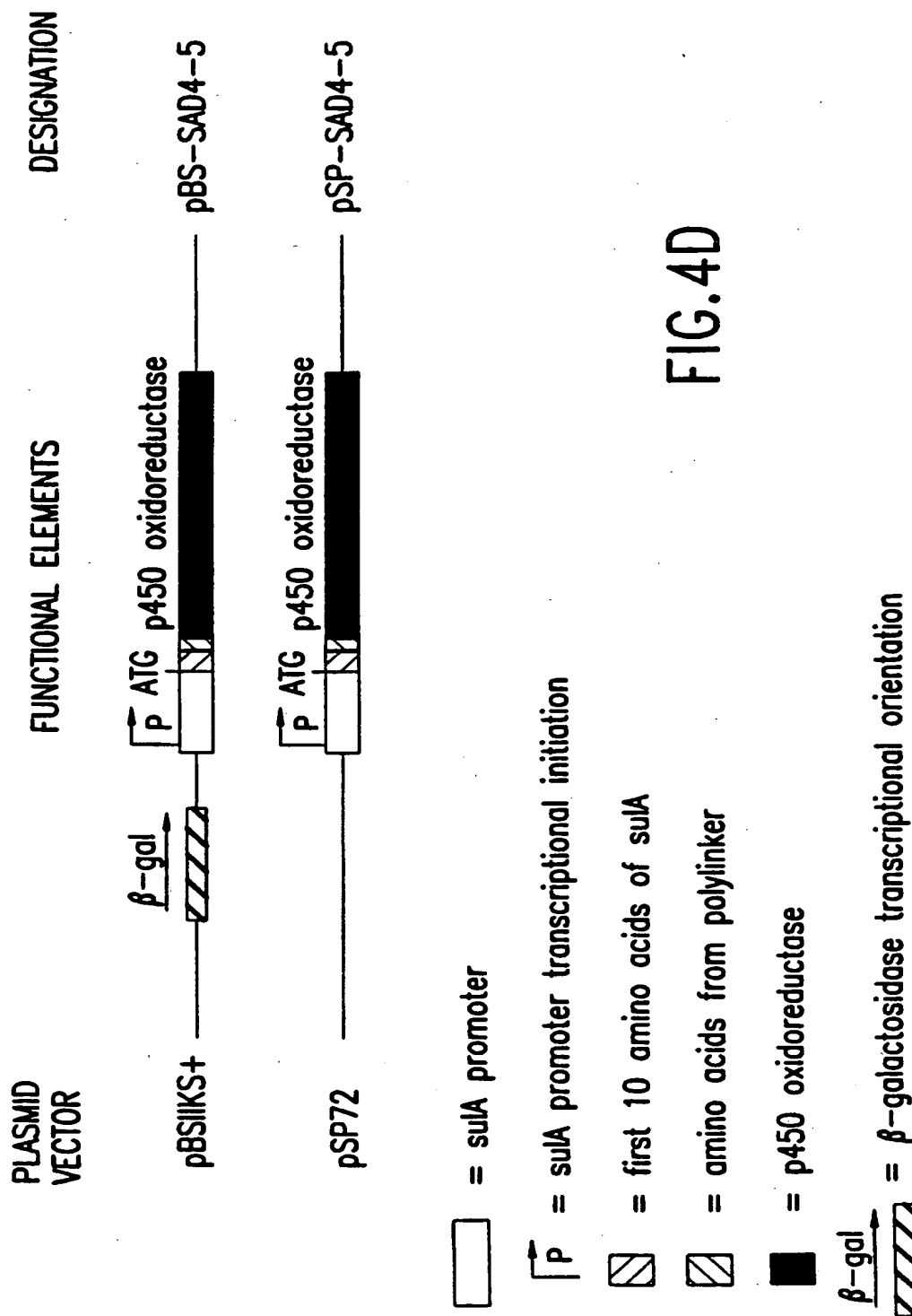
SUBSTITUTE SHEET (RULE 26)

FIG.4D
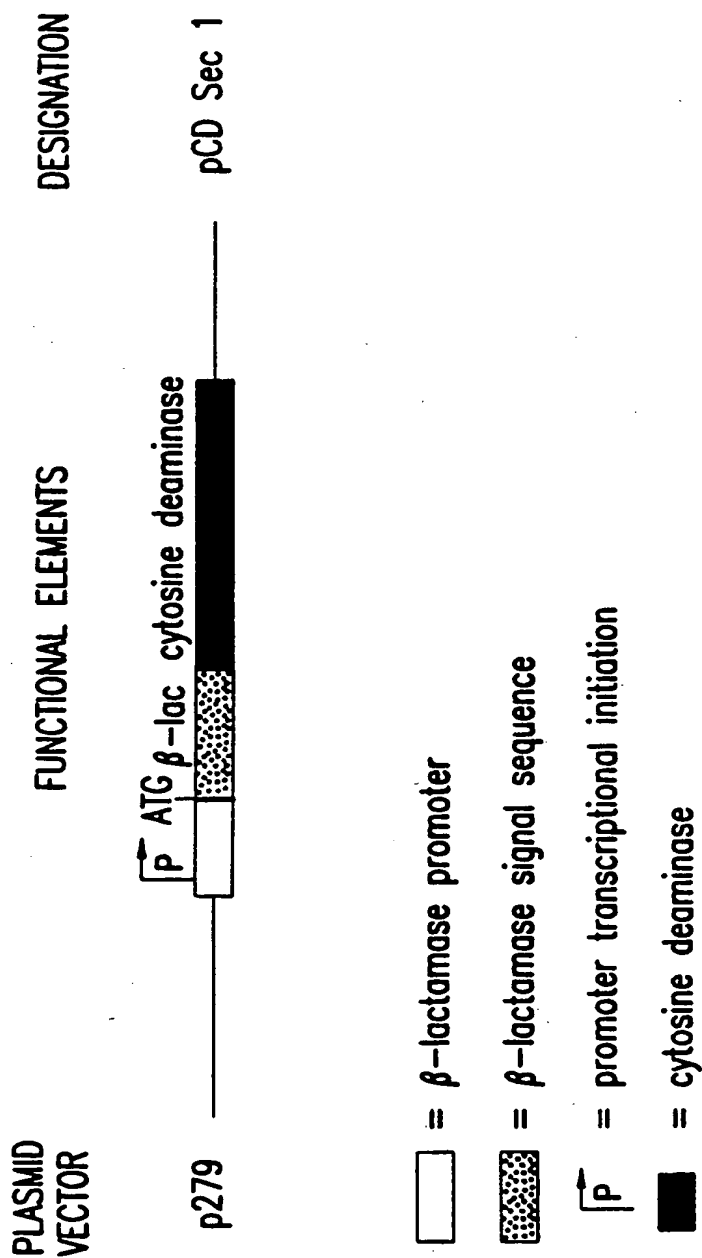
FIG.4E
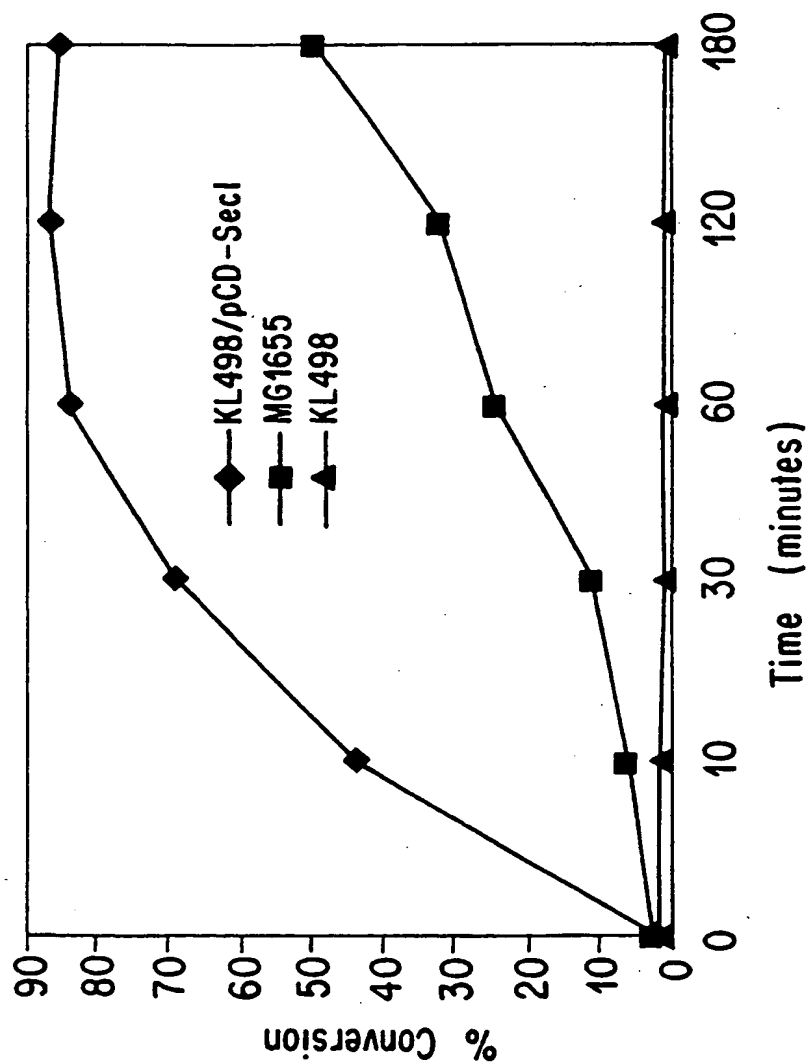
FIG. 4F
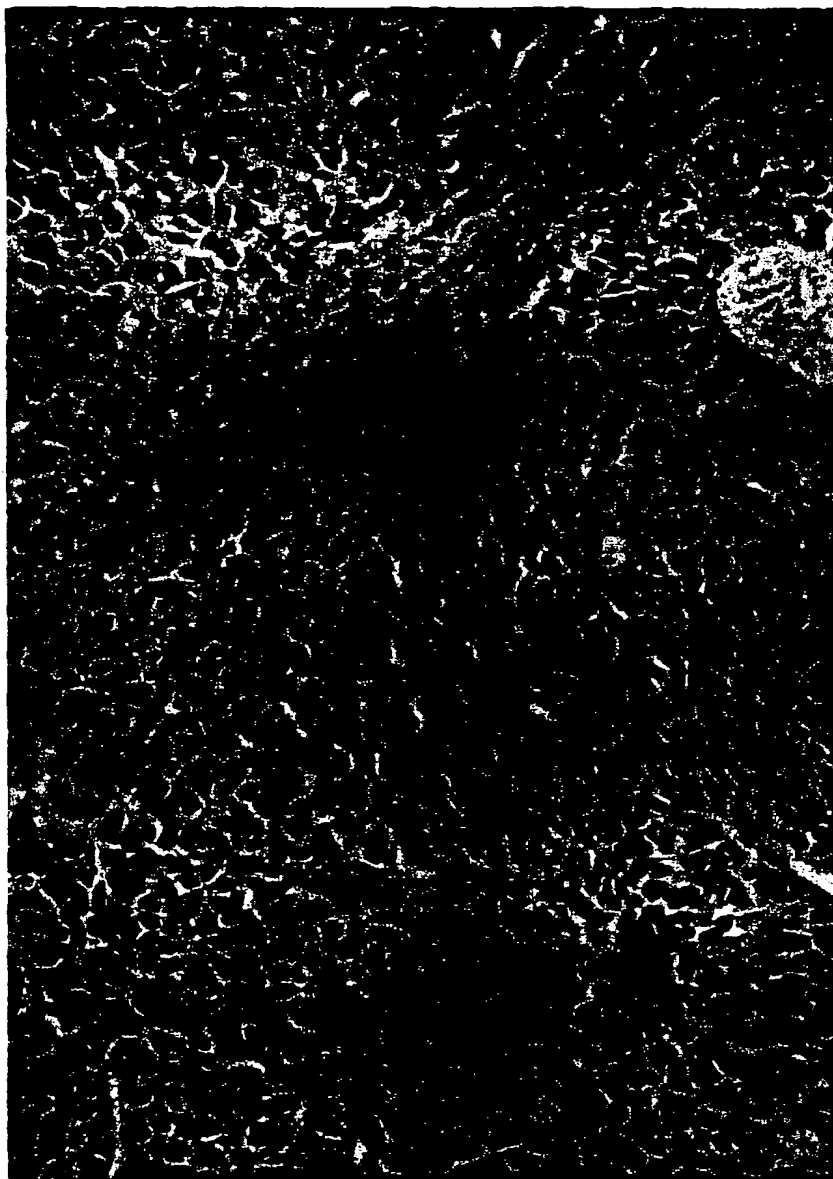
FIG.5A
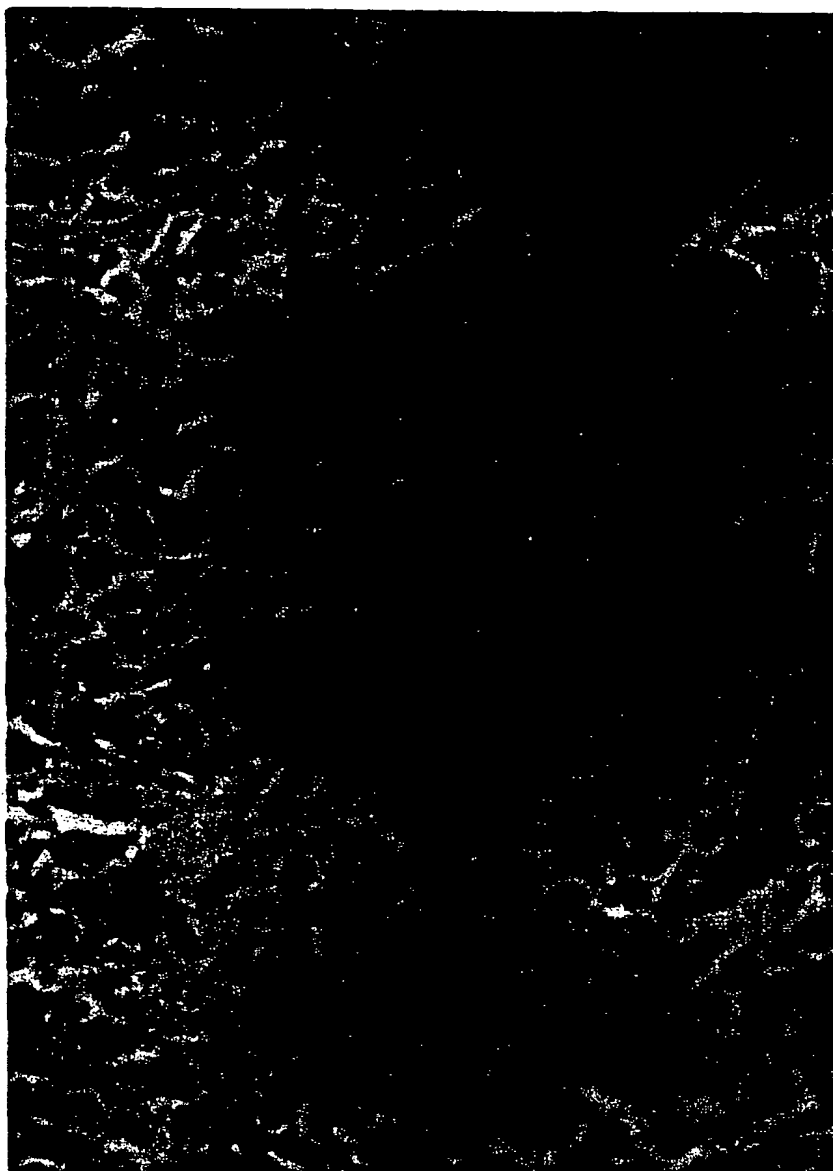
FIG.5B
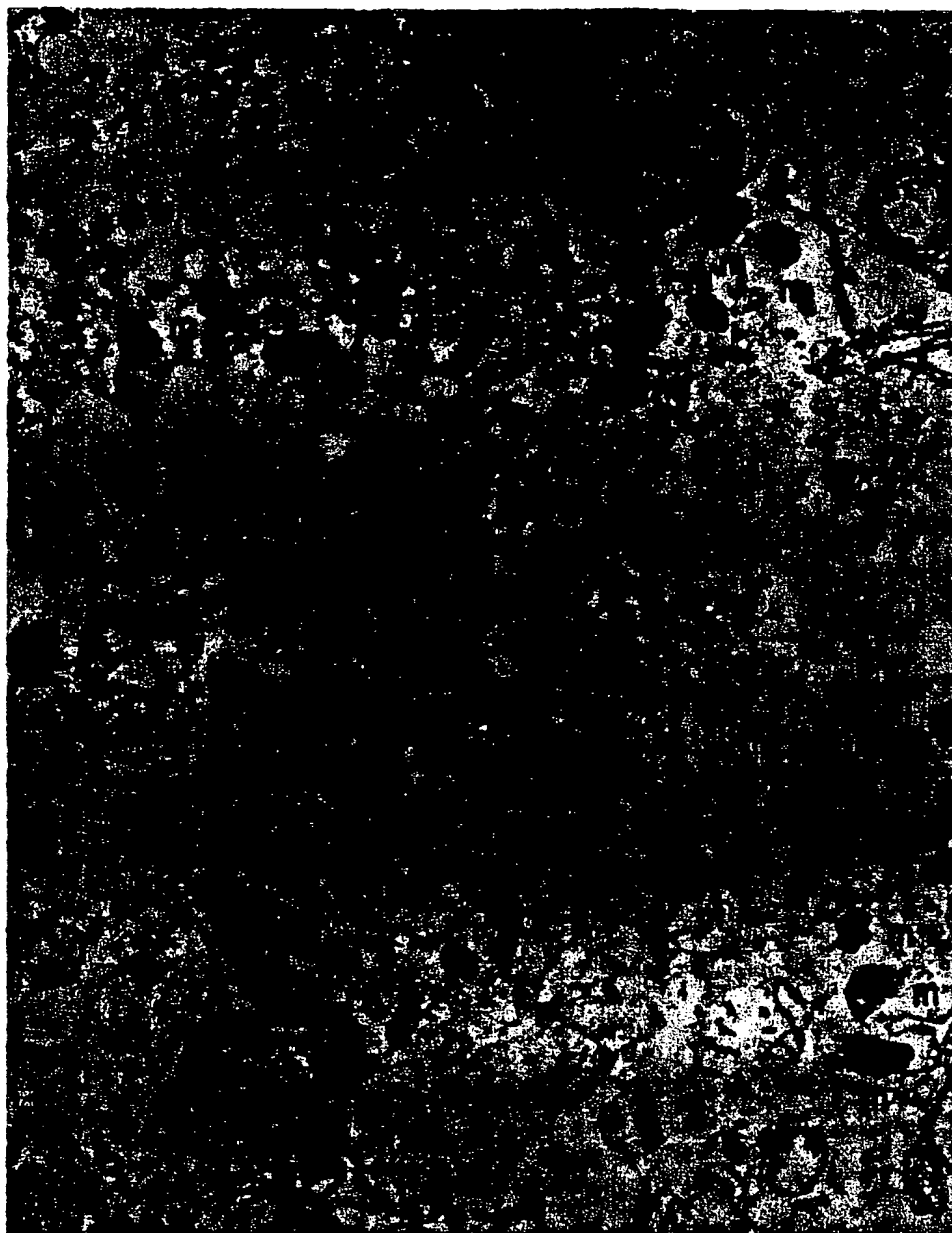
FIG.6

FIG.7
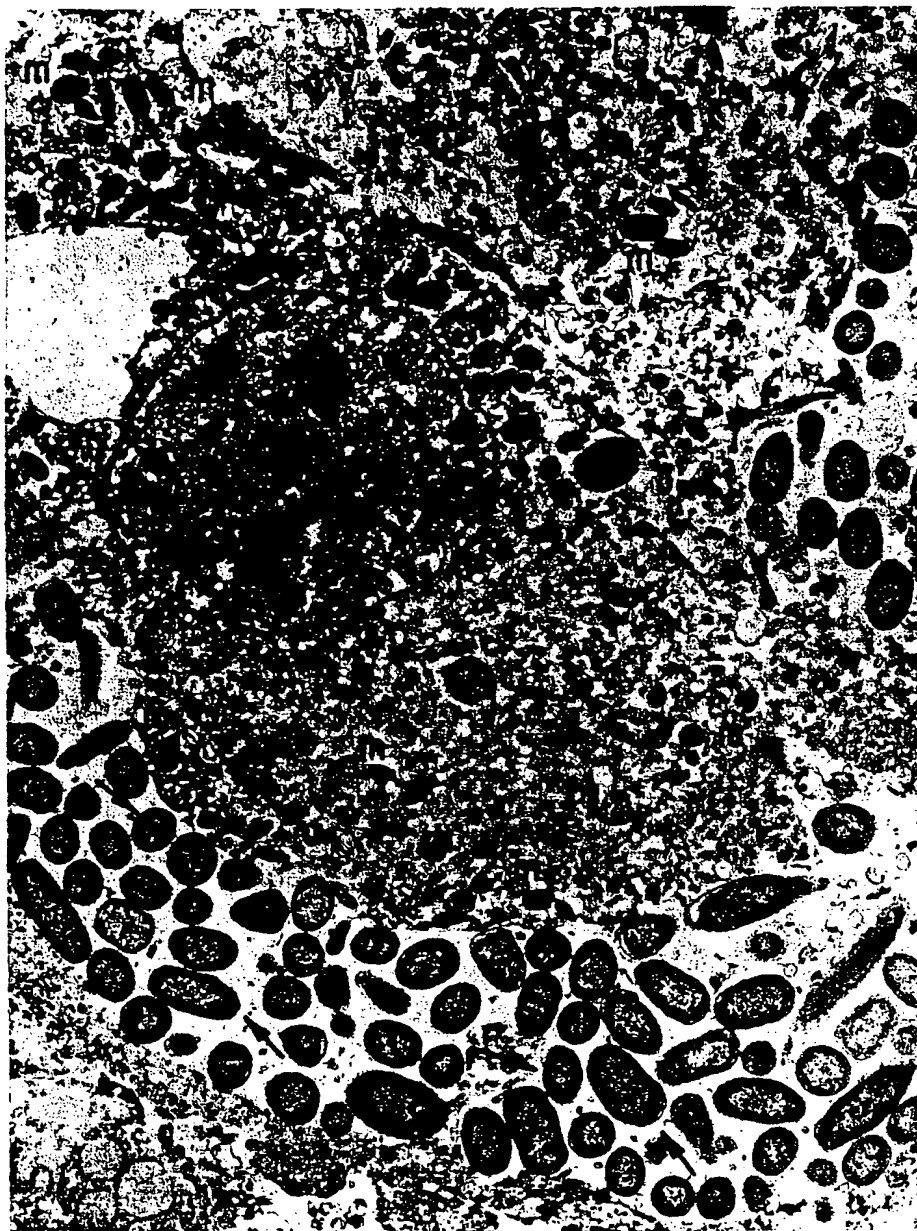
FIG.8

FIG. 9

CONTROL

FIG.10A

VECTOR (10 DAYS)
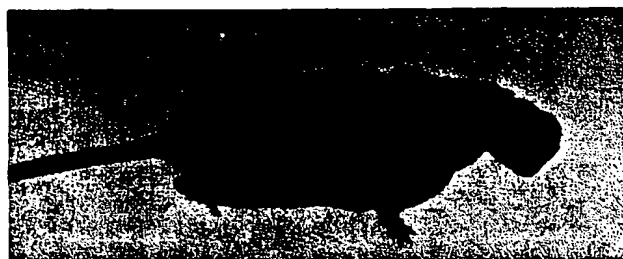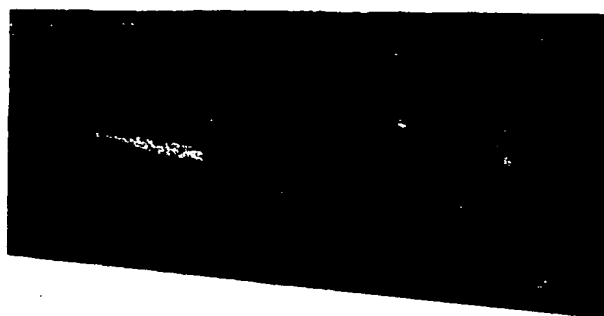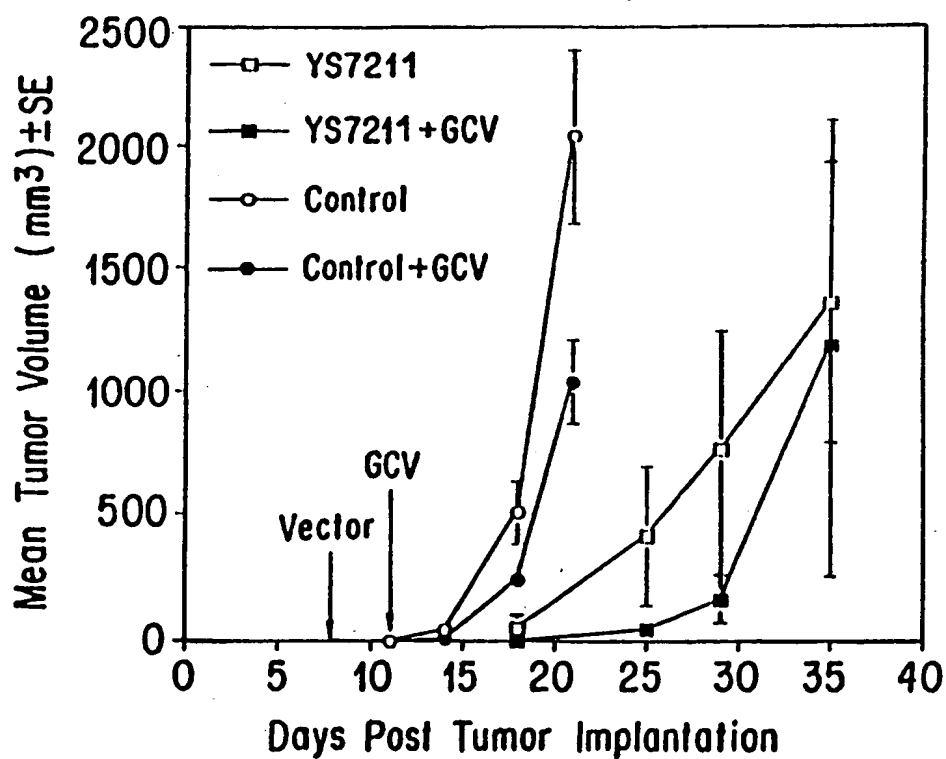
FIG.10B

CONTROL + GCV
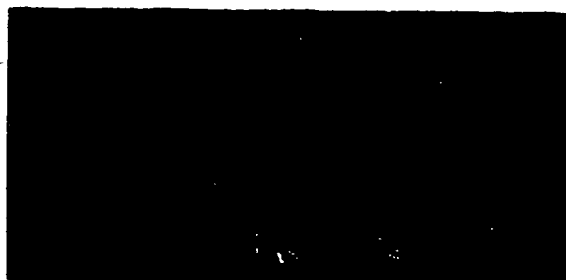
FIG.11A

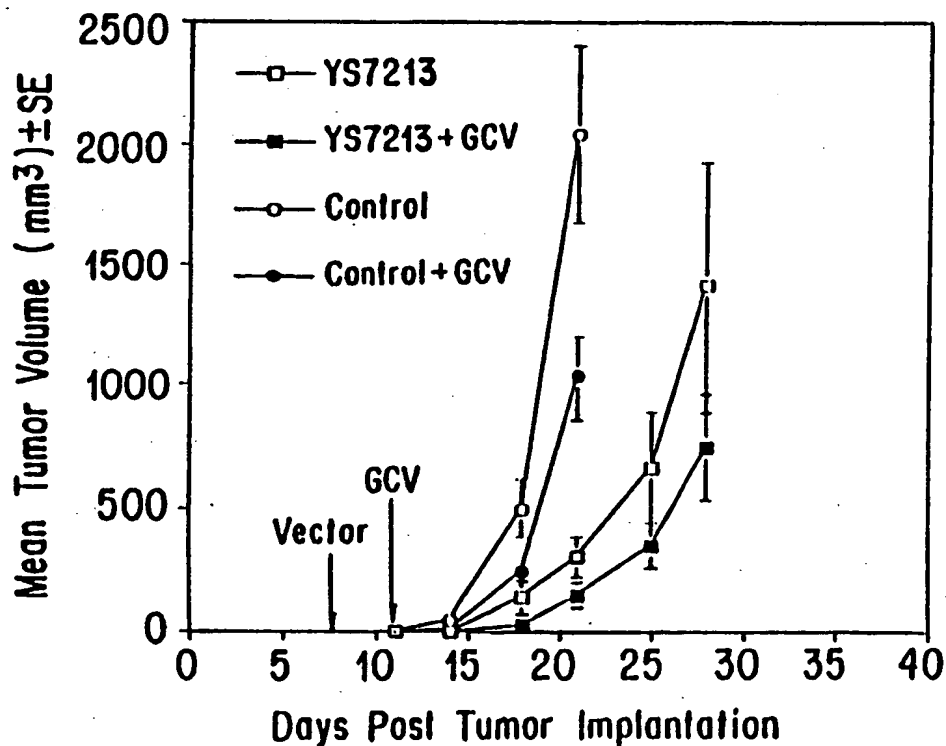TK VECTOR + GCV

FIG.11B

SUBSTITUTE SHEET (RULE 26)

FIG. 11C

FIG. 11D

FIG.11E